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CLINICAL AND EXPERIMENTAL

EXPERIMENTAL EVALUATION OF AN INDICATOR-DILUTION TECHNIQUE FOR ESTIMATION OF HEPATIC BLOOD FLOW

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BOSTON, MASS.

The use of the indicator-dilution principle for estimate of liver blood flow involves basic uncertainties traceable to variation in the anatomic extent of hepatic tissue drainage provided by the various hepatic venous branches, and random variation in the contribution to total liver blood flow provided by the portal vein and hepatic artery. When Bromsulphalein is used for this purpose, an additional defect is theoretically introduced in the method. This is traceable to the fact that the material used for indicator-dilution is also metabolized by the liver cells themselves. Variations in liver cell function may therefore introduce artifacts in the flow measurements.

To study these problems, the indicator-dilution principle has been used for measurement of the liver blood flow in dogs, with the use of radioactive chromated erythrocytes. By selective catheterization of both sides of the liver, it was possible to measure the homogenity, or lack thereof, in mixing and curve configuration between two large areas of hepatic tissue.

Comparison of the chromated red cell method with a modified Bromsulphalein method revealed an average mean deviation for the entire series of 8.5 per cent. When these comparisons included only those animals which fulfilled the conditions of bilateral symmetry, the agreement was within approximately 6 per cent.

It is concluded that under the conditions of these experiments in the dog the Bromsulphalein method yields a figure for liver blood flow which is corroborated by the use of an indicator independent of metabolic change.

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BROMSULPHALEIN[1-3] and colloidal chromium[4] are largely extracted in the liver by the hepatic and/or reticuloendothelial cells. The clearance rate of each is dependent upon the rate at which it is presented to the liver (flow), and upon the extracting capacity of the hepatic cells (function). Estimates of hepatic blood flow based on the clearance of Bromsulphalein (BSP) and colloidal chromium are complicated by these two variables (flow and function), either of which may change independently. Our purpose in exploring application of the indicator-dilution principle[5, 6] to the measurement of hepatic blood flow was to establish a method which was independent of hepatic function. Radioiodinated human serum albumin (RIHSA) was used as the indicator during the initial investigation and was later substituted by Cr^{51}-labeled autogenous erythrocytes.

Application of the Stewart-Hamilton indicator-dilution principle to this technique for estimating hepatic blood flow is based on 5 requirements[7, 8]: (1) that the indicator used not be metabolized by the liver; (2) that the known amount of indicator which is injected into the portal vein is not directed into the systemic venous system prior to its traversal of the liver; (3) that the injected substance be uniformly mixed in blood at the site of injection; (4) that the hepatic arterial-portal venous flow maintain a constant ratio of contribution to the total inflow of all parts of the liver during the period of observation. This requirement follows since blood enters the liver from hepatic artery as well as portal vein so that uniform mixing of the total inflow from a singular vascular injection is impossible; (5) that the volume of blood contained within the liver remain constant during the period of observation.

The previous studies of Haupt and associates[9, 10] and of Reichman and co-workers[11] have reported use of the indicator-dilution principle in the estimation of hepatic blood flow in dogs and man, respectively. In none of these reports, however, is there convincing evidence that the above-stated requirements have been fulfilled. In the present studies two simultaneous dilution curves, obtained from independent hepatic veins, were compared as an index of fulfillment of these requirements. The rates of hepatic blood flow calculated from these simultaneous dilution curves were compared, first with each other as an estimate of the accuracy of the method, and second with simultaneous estimates of hepatic blood flow by the BSP clearance technique.[1, 12] In this manner we have compared estimates of hepatic blood flow based on a clearance technique which is dependent, in part, upon hepatic function, with one which is independent of metabolic processes within the liver.

MATERIALS AND METHODS

Animal Preparation.—1. Acute experiments: Seven mongrel dogs weighing 14 to 18 kilograms were studied. Under Pentothal sodium anesthesia, with controlled positive pressure respirations, a midline thoracoabdominal incision was made. Retrograde polyethylene catheters (PE 240) were placed in the left common and right central hepatic veins through the anterior wall of the thoracic inferior vena cava. The spleen was removed and an injection catheter introduced into the portal vein through the divided splenic vein. A variety of injection catheters were used in these experiments. Initially, a polyethlene catheter (PE 205)

was introduced into the portal vein in the direction of flow. Subsequently, similar catheters were inserted retrograde into the vein. Finally, a No. 10 Fr. polyethylene catheter, with 9 orifices placed circumferentially at its tip, was introduced into the portal vein in the direction of the flow. In these acute preparations, the dilution curves were determined immediately upon completion of the operative procedure.

2. Chronic experiments: Five dogs, weighing 16 to 23 kilograms, were used in this portion of the study. Through a midline abdominal incision, a Genflex 607 catheter was placed in the left common hepatic vein by the method previously reported from these laboratories.[13] The central vein of the right central lobe of the liver was immediately lateral to the gall bladder; a similar catheter was introduced into it. The spleen was removed and a No. 10 Fr. polyethylene injection catheter was introduced into the portal vein in the direction of flow. The animals were allowed to recover for a period of 24 to 48 hours. Patency of the catheters was maintained by the intermittent infusion of heparin.

3. Protocol: The procedure for determination of hepatic blood flow by the indicator-dilution method was similar in its technical detail in both the "acute" and "chronic" experiments. In the first 6 "acute" experiments RIHSA was used as the indicator. In the final "acute" experiment, and in all of the "chronic" experiments, Cr^{51}-tagged autogenous erythrocytes were used.*

Each dog was heparinized (1 mg. per kilogram) during the experiment. Hepatic venous samples were collected fractionally at 2 second intervals. Flow in the catheters was maintained at a rate approximating 0.5 ml. per second by reversing the collecting tubing through a constant infusion pump. Ten milliliters of RIHSA (2.0 to 7.7 μc I^{131} diluted to 10 ml. in normal saline) or 20 ml. of labeled erythrocytes (12 to 25 μc of Cr^{51}) was injected rapidly (2 seconds) through the portal vein catheter and hepatic venous sampling begun at the mid-time of the injection. Twenty to 30 paired venous samples were collected. "Equilibration" samples were withdrawn at 10 and 20 minutes for determination of blood volume as a check against hemolysis of the injected erythrocytes. A 0.5 ml. aliquot of each hepatic venous sample was laked in 1.0 ml. of water and analyzed for radioactivity in a sodium iodide well-type scintillation counter.

In those "chronic" experiments where hepatic blood flow was determined by the BSP method, the dogs were placed in a Pavlov stand. A PE 90 catheter was introduced into a hind limb vein through a percutaneous needle. A priming dose of BSP (1 mg. per kilogram) was given, and a constant infusion of BSP begun at a rate of 0.07 to 0.09 mg. per kilogram per minute. One to 2 hours was allowed for equilibration of the plasma concentration. The actual measurement of flow was based on the analysis of from 3 to 12 paired samples (average, 6 pairs) obtained immediately before and after the dilution curves were measured.†

RESULTS

1. Acute Experiments.—A summary of the dilution curves observed in 7 "acute" experiments is presented in Table I. The significant points and time relationships of these curves are presented according to the scheme of Wood and Swan.[15] Representative paired curves are presented in Fig. 1. In only one of these acute experiments was a symmetrical pair of curves obtained

*Fifty milliliters of blood was withdrawn from the experimental animal at the beginning of the experiment. The erythrocytes were tagged with Cr^{51} by the method described by Read.[14] The injection dose of radioactivity was determined by analysis of duplicate aliquots of the injectate. Correction for that quantity of radioactivity remaining in the catheter at the end of the injection was determined by the withdrawal of a fixed volume of blood for analysis from the injection catheter at the end of the experiment.

†The analysis of BSP concentration in aqueous dilutions (3 : 1) of the plasma samples alkalinized to a pH greater than 10 with concentrated sodium hydroxide was carried out in a Beckman DU spectrophotometer at 580 μ. Correction for background color and for small amounts of hemolysis was based on an extrapolation of readings of these samples at 400 and 625 μ. The density of interfering substances at the 580 reading was determined from a previously prepared chart of the absorption spectra of alkalinized dilutions of hemolysed red cells in plasma from 500 to 625 μ.

(Experiment No. 6). In the remainder, sufficiently dissimilar dilution curves from either side of the liver were found to constitute convincing evidence that the requirements for the application of the indicator-dilution principle to the measurement of the hepatic blood flow had not been met. Thus, it was concluded that in the acute anesthetized preparation, immediately after retrograde catheterization of two hepatic veins and the portal vein, hepatic blood flow could not be reliably measured by the indicator-dilution principle.

TABLE I. SUMMARY OF DILUTION CURVES IN "ACUTE" EXPERIMENTS

EXPERIMENT NO.	INDICATOR	INJECTION OF CATHETER: TYPE	INJECTION OF CATHETER: HEPATIC VEIN USED	PEAK CONCENTRATION (COUNTS/MIN./1.0 ML.)	BUILD-UP TIME (SEC.)	PASSAGE TIME (SEC.)	HBF (ML./MIN.)
5	RIHSA*	PE 240	Right	55,580	16	†	†
			Left	8,068	34	†	†
6	RIHSA	PE 240	Right	39,835	6	46	361
			Left	37,000	8	56	349
7	RIHSA	PE 240	Right	50,022	4	†	†
			Left	16,300	8	†	†
8	RIHSA	PE 240	Right	54,315	8	50	737
			Left	39,058	6	52	1,173
9	RIHSA	10 Fr.	Right	17,830	14	†	†
			Left	34,359	10	72	294
10	RIHSA	10 Fr.	Right	6,463	4	†	†
			Left	3,407	24	†	†
12	Cr^{51} (R.B.C.)	10 Fr.	Right	2,485	12	†	†
			Left	6,989	10	70	307

Concentrations of radioactivity are expressed in counts per minute per milliliter. "Build-up time" is recorded as the duration of the upward slope of the dilution curve. The "passage time" is that interval occupied by the entire curve from its initial upward slope to the end of the first circulation on the extrapolated curve. "HBF" is the estimate of hepatic blood flow calculated from the dilution curve.

*Radioiodinated human serum albumin.

†Estimate of flow could not be calculated from these curves.

2. Chronic Experiments.—A summary of the simultaneous right and left hepatic venous dilution curves in the 10 "chronic" experiments is presented in Table II. Representative curves are presented in Fig. 2. From this summary, it is evident that the maximum concentrations of these simultaneous dilution curves were equivalent (± 10 per cent of the mean) in 8 of the 10 experiments. No explanation for the lack of symmetry between the two hepatic venous curves was evident in the experiments in which the curves were not symmetrical (Experiments 18-1 and 18-3). It was noteworthy that both of these paired observations occurred in one animal preparation which at autopsy had a small fibrin clot in the left hepatic vein catheter.

The build-up time (recorded from the initial rise to the greatest concentration) was similar in all 10 experiments. Contrasted with this observation was the dissimilarity in passage time (recorded from the initial upswing in concentration to the end of the first circulation on the extrapolated downward slope) between the simultaneous curves. Thus, in Experiments 16-2 and 17-2, there was pronounced dissimilarity in the duration of the simultaneous dilution curves from right and left hepatic veins. In neither of these 2 experiments, however, was there asymmetry in the peak concentrations.

In 6 of the 10 "chronic" experiments, symmetrical dilution curves from 2 independent hepatic veins were observed. When a flow rate was calculated from each curve, the resultant estimate of hepatic blood flow from either hepatic vein was found in essential agreement with its opposite (mean, ± 4.6 per cent standard deviation for the selected series in which comparable curves were found). The agreement from similar calculations in the whole series (including those in which asymmetry of the simultaneous curves occurred) revealed considerably less precision (mean, ± 9.6 per cent).

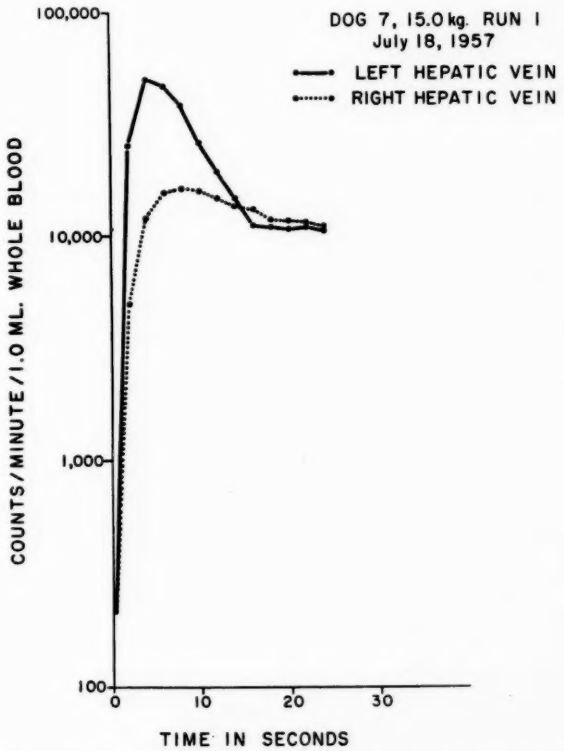

Fig. 1.—Simultaneous indicator (RIHSA) dilution curves from the "acute" experiments. The peak concentrations of these curves are significantly dissimilar. Similarly, if each is extrapolated, the passage time differs grossly between these curves.

In 4 of those experiments in which symmetrical dilution curves were obtained from right and left hepatic veins, hepatic blood flow was estimated simultaneously by the BSP method. A comparison of these independent estimates of flow is presented in Table III, which shows a close correlation between the results of those methods. Both methods were combined in an additional 5 experiments in which the indicator-dilution technique was considered unsatisfactory by virtue of a failure to demonstrate symmetry of 2 simultaneous curves. The results of these experiments are presented in Table IIIA.

"Equilibration" samples were obtained at 10 and 20 minutes after the injection of chromated erythrocytes for the determination of the whole blood volume. This determination varied from 5.6 to 8.2 per cent of body weight

TABLE II. SUMMARY OF DILUTION CURVES IN "CHRONIC" EXPERIMENTS

EXPERIMENT NO.	INDICATOR	HEPATIC VEIN USED	PEAK CONCENTRATION (COUNTS/MIN./1.0 ML.)	BUILD-UP TIME (SEC.)	PASSAGE TIME (SEC.)	HBF (ML./MIN.)
14-1	Cr^{51} (R.B.C.)	Right	8,956	8	48	505
		Left	7,934	8	54	505
14-2	Cr^{51} (R.B.C.)	Right	22,046	8	46	475
		Left	18,542	8	52	513
16-2	Cr^{51} (R.B.C.)	Right	5,140	10	62	1,069
		Left	4,664	8	38	661
16-3	Cr^{51} (R.B.C.)	Right	5,946	8	42	839
		Left	7,304	10	45	665
17-2	Cr^{51} (R.B.C.)	Right	5,012	10	48	1,182
		Left	4,290	12	74	1,005
18-1	Cr^{51} (R.B.C.)	Right	10,836	12	54	462
		Left	7,104	10	66	604
18-2	Cr^{51} (R.B.C.)	Right	4,898	8	44	1,090
		Left	5,428	8	42	954
18-3	Cr^{51} (R.B.C.)	Right	3,462	8	46	1,099
		Left	5,930	8	42	728
20-1	Cr^{51} (R.B.C.)	Right	6,550	6	32	871
		Left	6,096	6	30	1,045
20-2	Cr^{51} (R.B.C.)	Right	4,664	6	26	1,473
		Left	5,652	4	22	1,478

Concentrations of radioactivity are expressed in counts per minute per milliliter. "Build-up time" is recorded as the duration of the upward slope of the dilution curve. The "passage time" is that interval occupied by the entire curve from its initial upward slope to the end of the first circulation on the extrapolated curve. "HBF" is the estimate of hepatic blood flow calculated from the dilution curve.

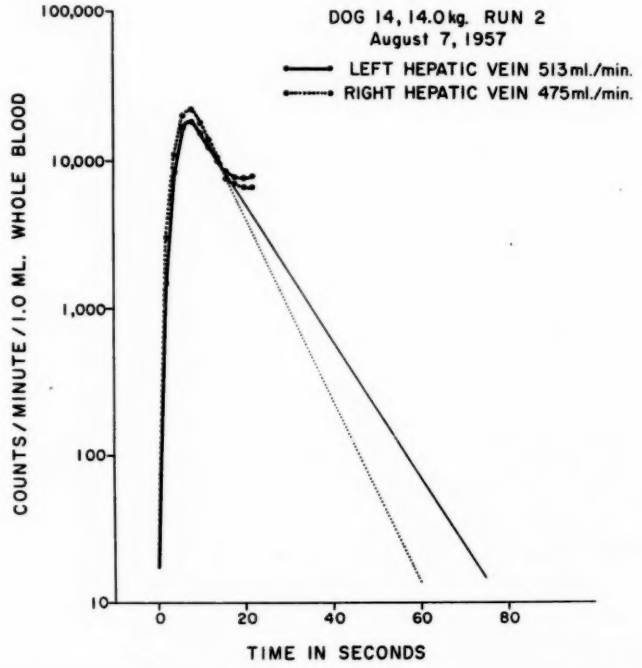

Fig. 2.—Simultaneous indicator (Cr^{51} R.B.C.) dilution curves from the "chronic" experiments. These curves are sufficiently similar to warrant a calculation of hepatic blood flow by the indicator-dilution principle.

(mean, 6.4 per cent) for this series of animals, a figure which correlates closely with results determined from a parallel series of normal animals studied in this laboratory.[16] We therefore believe that no significant hemolysis of the injected erythrocytes occurred in these experiments.

TABLE III. COMPARISON OF ESTIMATES OF HEPATIC BLOOD FLOW DETERMINED BY THE BROMSULFALEIN (BSP) METHOD AND THE INDICATOR-DILUTION TECHNIQUE

EXPERIMENT NO.	HBF* (Cr^{51}) (ML./MIN.)		ARTERIOVENOUS HBF (Cr^{51}) (ML./MIN.)	HBF (BSP) (ML./MIN.)
16-3	Right	839	752	837
	Left	665		
18-2	Right	1,090	1,022	1,200
	Left	954		
20-1	Right	871	958	1,130
	Left	1,045		
20-2	Right	1,473	1,476	1,670
	Left	1,478		

This series consists of those experiments in which, by virtue of similarity between the simultaneous dilution curves, this estimate of flow was considered valid.

*"HBF" is the estimate of hepatic blood flow calculated from the dilution curve.

TABLE IIIA. COMPARISON OF ESTIMATES OF HEPATIC BLOOD FLOW DETERMINED BY THE BROMSULFALEIN (BSP) METHOD AND THE INDICATOR-DILUTION TECHNIQUE

EXPERIMENT NO.	HBF* (Cr^{51}) (ML./MIN.)		ARTERIOVENOUS HBF (Cr^{51}) (ML./MIN.)	HBF (BSP) (ML./MIN.)
16-1	Right	583	583	575
	Left	†		
17-2	Right	1,182	1,094	1,050
	Left	1,005		
18-3	Right	1,099	914	1,250
	Left	728		

This series consists of those experiments in which the estimate of flow by the indicator-dilution technique was not satisfactory.

*"HBF" is the estimate of hepatic blood flow calculated from the dilution curve.

†Sample clotted.

DISCUSSION

After an initial evaluation of this technique, utilizing I^{131}-labeled serum albumin, chromated erythrocytes were chosen as the indicator in the "chronic" experiments to preclude an effect of hepatic metabolism on the resultant dilution curves. Provided that no hemolysis of the injected indicator occurred, the resultant estimate of flow made by this indicator should be independent of hepatic function.* The calculation of blood volume in these experimental animals was not dissimilar to that made in a parallel series of dogs studied in this laboratory. Therefore, we concluded that no significant hemolysis of the injected erythrocytes occurred, and, hence, that the indicator fulfilled the requirement of independence from hepatic metabolism.

*The occurrence of hemolysis could, in this instance, lead to two sources of error: (1) That labeled cell fragments are rapidly extracted from the circulation may lead to an erroneous value for the injected dose of indicator in the eventual calculation of the volume of blood flow. (2) Adenosinetriphosphate (ATP) released from the hemolysed red cells may, in itself, lead to significant changes in flow. Sharp increases in flow rates in human extremities have been documented coincidentally with the local infusion of minute quantities of ATP. This subject has been admirably reviewed by Andres and associates.[7]

Extrahepatic diversion of the injected indicator prior to its traversal of the hepatic circulation was precluded by meticulous ligation of all branches of the portal vein between the injection catheter and the hilus of the liver. After assurance that all of the injected indicator would traverse the hepatic circulation and that hepatic metabolism would not influence its behavior in that circulation, the resultant time-concentration curves from hepatic vein samples should allow for calculation of the hepatic blood flow, provided that the indicator was equally distributed in all parts of the liver relative to the rate of blood flow in each part. Equal distribution of the indicator in blood requires not only that the indicator be equally mixed in venous blood at the site of portal injection, but further that labeled portal vein blood be proportionally distributed in the liver with unlabeled blood from the hepatic artery. Further, it was reasoned that changes in the volume of blood contained within hepatic parenchyma, were it to change during the course of the experiment, might lead to alterations in the dilution curves which would be difficult to interpret. Accompanying decreases in hepatic volume, the indicator should be further diluted by that quantity of blood discharged from the parenchyma. In the case of increases in this residual volume, a quantity of indicator could remain "trapped" within the liver. In neither instance, then, could the indicator-dilution estimate be considered synonymous with blood flow in its strictest sense (i.e., that volume of blood traversing the organ during the period of observation).

Recognition of these requirements leads us to feel that any estimate of blood flow utilizing the indicator-dilution principle should be considered suspect until it can be demonstrated that these requirements have been fulfilled under the circumstances of the particular experiment. Should these requirements be met, dilution curves from any of the hepatic veins would be similar. Similarity might occur as a result of fortuitous synchronous deviation of two of these variables. Such chance occurrence should be incurred only rarely, however, and therefore, the repeated demonstration of symmetrical simultaneous curves from two independent hepatic veins may afford an index of the validity of the indicator-dilution principle in the estimation of hepatic blood flow.

In only one of the 7 "acute" experiments was a symmetrical pair of dilution curves observed. Symmetrical simultaneous dilution curves were seen in 6 of the 10 "chronic" experiments. In the remainder, either as a result of inequality of the peak concentrations or of the duration of the primary curve, the calculation of hepatic blood flow based on samples from one hepatic vein was, at best, tenuous. This dissymmetry apparently occurred in the "acute" experiments irrespective of the characteristics and position of the portal venous injection catheter. Further, the markedly contrasting results between the "acute" and the "chronic" experiments led us to feel that factors other than equal mixing of the injected indicator in portal venous blood were primarily responsible for the failure to obtain similar simultaneous curves.

In considering other possible explanations for the observed results of these experiments, one gross observation seems worthy of note. During the course of each of the "acute" experiments, progressive congestion of the liver,

manifested by darkening and swelling, was observed. This phenomenon was pronounced, often asymmetrical, and was interpreted as a manifestation of significant increase in the volume of blood contained within hepatic parenchyma. This phenomenon was apparently similar to that reported by Kestens, Austen, and McDermott[17] in the perfused canine liver, which was interpreted by them as "outflow block" of the liver. Whether hepatic venous obstruction is the whole explanation of this lesion, or whether it is associated with change of the portal venous and hepatic arterial circulation, is unknown. At least its uniform occurrence in the present "acute" experiments was associated with failure to obtain satisfactory estimates of the hepatic blood flow by the indicator-dilution principle.

Avoidance of this "outflow block" during perfusion of the canine liver has been found to depend upon avoidance of blood loss, trauma, hypoxia, hypotension, etc.[17] The markedly contrasting results in the "acute" and "chronic" experiments in this study suggest at least that the effects of anesthesia and surgical operation in the "acute" preparations adversely influence the results of the immediate estimates of flow. It seems likely that this adverse influence on the dilution curves manifests a change in the dynamics of hepatic circulation similar to, if not the same as, this "outflow block" phenomenon.

It was noted, however, that even in the "chronic" animal preparations, hepatic blood flow could not be estimated uniformly by the indicator-dilution principle. Within the limits imposed by the presence of chronic inlying portal and hepatic vein cannulas, these animals were in as nearly a normal state as could be achieved. Consistently reliable estimates of flow were not obtained by the dilution technique even in this circumstance. For this reason, it is felt that the indicator-dilution principle does not uniformly afford a technique for estimating blood flow in the laboratory.

In those instances wherein the requirements set forth were met, the indicator-dilution principle which uses Cr^{51}-labeled erythrocytes served as a reliable estimate of hepatic blood flow which coincided closely with simultaneous estimates of flow based on the metabolism-dependent BSP method.

SUMMARY

An evaluation of the indicator-dilution technique for estimation of hepatic blood flow in the dog is reported. Iodinated albumin, or Cr^{51}-labeled erythrocytes, was used in the indicator, and injected directly into the portal vein. Simultaneous dilution curves were obtained from 2 independent hepatic veins, as an estimate of the validity of this technique.

1. Symmetrical dilution curves were obtained in only one out of 7 "acute" experiments carried out (immediately after operative insertion of hepatic venous sampling catheters and the portal vein catheter).

2. Similar curves were obtained from 2 hepatic veins in 6 of 10 "chronic" experiments (24 to 48 hours after the insertion of the catheters).

3. In 4 of the "chronic" experiments in which symmetrical dilution curves were obtained, simultaneous estimates of hepatic blood flow by the

Bromsulphalein technique were made. Good agreement between these estimates was observed under these conditions.

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ROLE OF THE LIVER DURING THE PROTECTIVE ACTION OF ARGININE

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Arginine has been administered to patients with "liver insufficiency" because it has been shown to prevent acute ammonia intoxication in experimental animals. It is open to question if an "insufficient liver" can utilize arginine as does a normal liver. The reported data obtained on rats showed that: (a) A low but chronic hyperammoniemia nullified the protective action of arginine against acute ammonia intoxication. (b) Restoration of the protective action of arginine was achieved by the injection of liver homogenates. (c) During acute ammonia poisoning free arginine of the hepatic blood decreased more rapidly in the animals protected with arginine than in unprotected animals. The route of administration of the ammonia donor was a critical factor in reducing the utilization of arginine by the liver. The protective action of arginine did not necessarily either occur or improve when the concentration of free arginine in the hepatic blood was raised. (d) Small doses of arginine increased the over-all excretion of urea in partially hepatectomized rats with high hyperammoniemia.

EARLIER studies have demonstrated that under certain conditions L-arginine · HCl was able to ensure survival of all rats given a lethal dose of ammonium acetate or an amino acid mixture.[1, 2] The lethal effect of these compounds is generally ascribed to their property of increasing rapidly the level of free blood ammonia. In rats poisoned with ammonia, arginine was able to lower free blood ammonia and increase the amounts of urea excreted.[3] The best results were obtained when arginine was brought directly to the liver by intrasplenic or intraportal administration.[4] On the assumption that arginine stimulated urea formation in vivo and, therefore, might benefit human patients with liver damage, arginine was given to patients in "hepatic coma."[12-17] In some cases[15] the effect was good, in others[13] there was no effect. The over-all clinical experience suggested that more had to be learned about the role played by the liver on the protective effect of arginine during ammonia intoxication. In order to investigate this problem in more detail, the protective effect of arginine was studied in experimental animals in the following ways: (a) when acute poisoning was obtained with different ammonia-producing compounds given through different

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routes of administration; (b) when rats were kept under chronic hyperammoniemia; (c) when various levels of free arginine were present in the hepatic blood during acute ammonia intoxication; and (d) when hepatectomized rats were treated with arginine and subjected to a chronic hyperammoniemia. The results of these studies are presented in this paper.

MATERIALS AND METHODS

About 2,000 rats of the Sprague-Dawley, National Institutes of Health strain, which weighed between 180 and 210 grams, were employed. The animals, raised on a Purina Chow diet, were deprived of food but given water ad libitum 24 hours prior to study. When the influence of arginine on urea production was determined in rats with chronic hyperammoniemia, the animals were fed for one week before any experiment a synthetic water soluble diet[5] either with all essential amino acids or deprived of arginine.* The quantity of synthetic diet was limited to 22 ml. per day per 200 gram rat weight corresponding to an intake of 290 mg. nitrogen and 44 calories. Normally, a 200 gram rat consumes 25 to 28 ml. of diet per day, but a restriction of 22 ml. did not change appreciably the growth curve. However, this restriction resulted in a constant and comparable food intake, since the normal animal always consumed 22 ml. of diet and the level of urea excretion in the same or different rats was more constant. In order to stabilize the urea excretion following a change in experimental procedure, it was necessary to keep the rats in metabolism cages for at least one week.

Hepatectomy was performed under ether anesthesia by excision of the central and left lobes of the liver. The average of liver removed was 5.4 ± 0.7 grams, or about 60 to 70 per cent of the total liver of a 200 gram rat.[6]

Blood was drawn from the hepatic veins as follows: The rat was anesthetized with ether. A catheter (polyethylene tubing PE 50),† introduced into the right jugular vein, was passed through the inferior vena cava, and, after laparotomy, guided into a hepatic vein. The quantity of blood removed each time varied between 0.5 and 1.0 ml.; the total amount never exceeded 3.0 ml. The blood sample was immediately mixed with ethyl alcohol to a final alcohol concentration of 80 per cent. After about 15 minutes the mixture was centrifuged. The supernatant was dried completely, then redissolved in 80 per cent alcohol for chromatography.

Chromatography was carried out as reported by DuRuisseau and associates.[7] The position of the arginine spot was checked by Sakaguchi's test and citrulline was identified by Ehrlich's reagent.[8]

The dose that would kill 99.9 per cent of the rats ($LD_{99.9}$) was determined for ammonium acetate, ammonium chloride, and isonicotinic acid hydrazide following a procedure previously described.[2] These compounds were given in 2 ml. water solutions and $LD_{99.9}$ values corresponding to oral, subcutaneous, and intraperitoneal routes of administration were calculated.

The protective action of L-arginine · HCl, L-ornithine, and L-citrulline were determined at levels ranging from 1.0 to 8.0 mM. per kilogram in 1 ml. water solution. The protective agents were administered subcutaneously, orally, intraperitoneally, intravenously, or directly into the spleen either simultaneously or at various times (30 to 120 minutes) prior to the ammonia donor. A chronic hyperammoniemia was obtained by the feeding of the ammonium salt of Dowex-50 × 8 twice a day.‡ The resin was prepared by treating the hydrogen form with 2N ammonium hydroxide and then washing with water until the effluent was neutral. One gram of partially dried resin released, on the average, 22.0 mg. of ammonia

*The amino acids used in the following experiments were provided by the late Dr. J. P. Greenstein. The methods of preparation and the degree of purity have been reported in Arch. Biochem. 64: 342, 1956.

†Clay Adams, New York, N. Y.

‡Dow Chemical Company, Midland, Mich.

on elution with 1N HCl. The appropriate dose of resin was force-fed by stomach tube in a slurry with 3.0 ml. water. The quantity of ammonia administered to each rat was 32 mEq. per kilogram per 24 hours. About one hour after the ingestion of resin the blood ammonia level rose to 10 to 15 μg per milliliter and remained at this level for weeks, resulting in the excretion of increased amounts of urine and urea, but without any clinical damage to the rats. To counteract the resin-induced acidosis, sodium and potassium bicarbonate were given by stomach tube or by intraperitoneal injection in 2 ml. water solution. Another procedure was also used to achieve a higher level of prolonged ammoniemia. Cellulose tubing* (about 1.5 by 0.6 cm.) loaded with 100 mg. ammonium acetate and 200 mg. Solka-Floc,† was tied at the ends and introduced into a pouch of the subcutaneous tissue of the interscapular region. It was thus possible to maintain the blood ammonia level at a range varying from 20 to 30 μg per milliliter. By renewal of the preparation every 2 hours a high blood ammonia level could be maintained for 8 hours.

The total phosphorus in the liver parenchyma was determined by the method of Boltz and Mellon,[9] and potassium, by the ultramicro procedure of Caraway and Fanger[10] after combustion and solution of ash.

Urine was collected under toluene and acidified with 6N HCl. Urea and ammonia in urine were determined by the urease and aeration methods, ammonia in blood or in serum by the Conway method.[11]

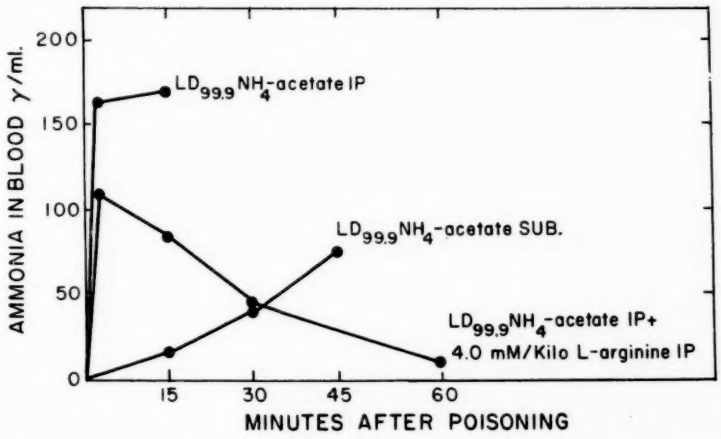

Fig. 1.—Free ammonia in blood serum during poisoning with ammonium acetate and protection with arginine. *IP* = intraperitoneally and *SUB.* = subcutaneously.

RESULTS

Range of Arginine Protective Effect.—The protective action of L-arginine · HCl and the related compounds L-ornithine and L-citrulline was tested in rats poisoned with lethal doses of 3 different ammonia donors: ammonium acetate, ammonium chloride, and isonicotinic acid hydrozide. The doses that killed 99.9 per cent ($LD_{99.9}$) of the experimental rats were calculated following the oral, intraperitoneal, or subcutaneous route of administration of the ammonia donor. The amounts of "free ammonia" in the peripheral blood were determined at death and during poisoning. The percentages of rats surviving the lethal doses after treatment with arginine, ornithine, or citrulline were calculated, and the

*Visking Corporation, Chicago, Ill.
†Solka-Floc BW 40, Brown Company, Berlin, N. H.

TABLE I. EFFECTIVENESS OF L-ARGININE · HCl AGAINST POISONING WITH DIFFERENT AMMONIA DONORS

AMMONIA DONORS	ROUTE OF ADMINISTRATION	$LD_{99.9}$ (mM./Kg.)	FREE NH_3 IN SERUM AFTER $LD_{99.9}$ (γ/ML.)*	PER CENT SURVIVING AFTER TREATMENT†		SURVIVAL TIME AFTER $LD_{99.9}$ (IN MINUTES)‡	
				L-ARGININE · HCl	L-ORNITHINE OR L-CITRULLINE	WITHOUT L-ARGININE · HCl	WITH L-ARGININE · HCl
NH_4 acetate	Intraperitoneal (IP)	10.8	172	100§	100‖	15	360¶
	Subcutaneous	37.0	75	10**	0	44	50
	Oral	72.6	168	0	0	13	15
NH_4 chloride	Intraperitoneal	15.1	165	100§	100‖	13	360¶
	Subcutaneous	60.0	67	0	0	30	30
	Oral	76.1	170	0	0	13	18
Isonicotinic acid hydrazide	Intraperitoneal	3.2	35	0	0	60	65
	Subcutaneous	3.0	28	25††	0	45	60
	Oral	8.1	40	20††	0	45	50

*Physiologic values 1 to 2 γ per milliliter. The reported numbers are averages on groups of 5 to 8 rats. The extreme values agree within ± 15 per cent. Blood withdrawn during terminal convulsions.

†Each amino acid given intraperitoneally, subcutaneously, orally, and intravenously in doses ranging from 1.0 to 8.0 mM. per kilogram.

‡The values reported are means from groups of 20 to 40 animals. The extreme values agree within ± 20 per cent.

§One hour prior to $LD_{99.9}$; 4.0 mM. per kilogram IP.

‖One hour prior to $LD_{99.9}$; 8.0 mM. per kilogram IP.

¶Animals surviving 360 minutes after treatment had the normal life span.

**2.0 or 4.0 mM. per kilogram IP 30 minutes prior to $LD_{99.9}$.

††8.0 mM. per kilogram IP 30 minutes prior to $LD_{99.9}$.

surviving times of protected and unprotected animals were recorded. The results are summarized in Table I. The $LD_{99.9}$ varied not only with the route of administration but also with the ammonia-forming compound. In some instances, these differences were surprisingly large. The blood ammonia levels at death were also divergent. The protective action of arginine, ornithine, or citrulline was complete when ammonium salts were injected intraperitoneally, but was totally lacking when the lethal dose was given orally or subcutaneously.

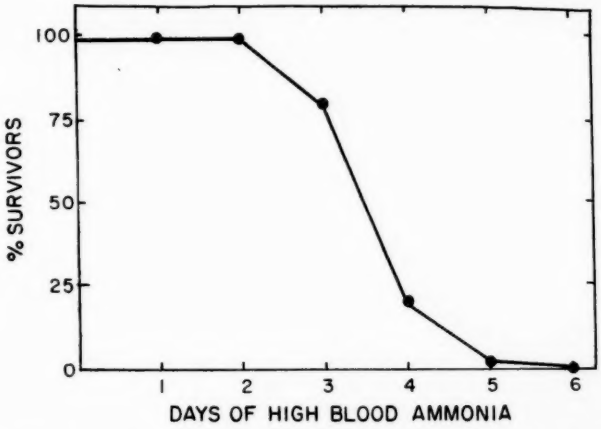

Fig. 2.—Effectiveness of L-arginine·HCl in protecting rats during chronic ammonia poisoning. Each point refers to 20 rats bearing a hyperammoniemia of 10 μg per milliliter and challenged with a lethal dose of ammonium acetate after receiving a protective dose of arginine. The complete protection afforded by arginine to normal rats was not modified by 2 days of hyperammoniemia but was nullified after 6 days.

On the other hand, although arginine showed some protective effect against oral or subcutaneously administered isonicotinic acid hydrazide, none was obtained on intraperitoneal administration. There was no significant increase in survival time whenever protection was absent, but the survival time was longer when free blood ammonia was lower. Fig. 1 shows that the route of administration influences the degree and duration of hyperammoniemia. The intraperitoneal lethal dose of ammonium acetate produced an increase of free blood ammonia to values more than 150 times the normal in a few minutes; the animal died shortly thereafter. The protective dose of arginine limited the initial increase and produced a decrease to physiologic values in about one hour. The subcutaneous lethal dose, on the contrary, produced a slower but steady increase of free blood ammonia which could not be stopped by any dose of arginine, ornithine, or citrulline, whether injected before or during poisoning. At death, these animals had a level of free blood ammonia of about 75 μg per milliliter lower than the levels found in rats protected by arginine after the intraperitoneal lethal dose. Rats receiving an intraperitoneal $LD_{99.9}$ would have survived with such a blood level of free ammonia. It appeared that the prolonged duration of the elevated free NH_3 level in the blood of rats receiving the subcutaneous $LD_{99.9}$ might explain the failure of arginine to prevent death. In order to explore the possibility further, the effect of chronic hyperammoniemia was studied.

Effect of Chronic Hyperammoniemia.—By the feeding of NH_3-donor, resin-free blood ammonia was artificially increased and kept at 10 μg per milliliter* in 6 groups of 20 rats each. At different intervals the animals of each group were injected intraperitoneally with the protective dose of arginine and, one hour later, with the intraperitoneal lethal dose of ammonium acetate. All animals survived if the hyperammoniemia lasted for only one or two days; if it lasted longer the protective action of arginine sharply decreased and at the sixth day was absent (Fig. 2). This experiment was repeated with two changes in the procedure. The first change consisted in the counteraction of the acidosis produced by the ammonia donor resin with sodium and potassium bicarbonate, given orally. The results were similar to those shown in Fig. 2. The second change consisted in the increase of the ammoniemia at about 25 μg per milliliter. In this instance arginine provided complete protection for only the first 2 hours, thereafter, the protective effect decreased, and was absent at the eighth hour. The curve obtained was very similar to that shown in Fig. 2, if the time scale is expressed in hours instead of days (Fig. 3).

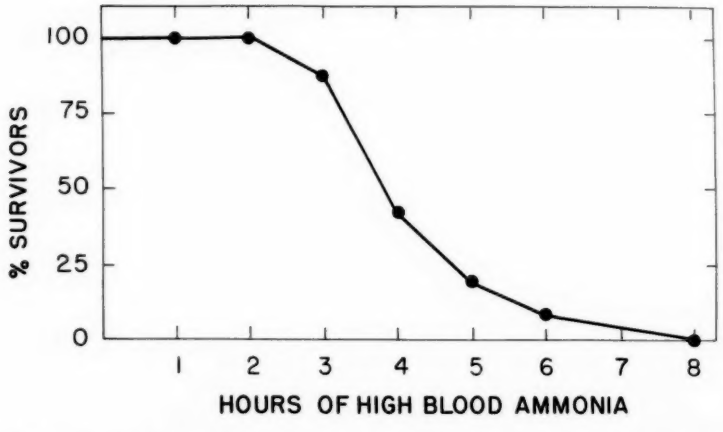

Fig. 3.—Each point refers to 10 rats bearing a hyperammoniemia of 25 μg per milliliter and challenged with a lethal dose of ammonium acetate after receiving a protective dose of arginine. Eight hours were sufficient to nullify the protective effect of arginine.

In another experiment 20 rats were force-fed for 2 months with a quantity of ammonia donor resin sufficient to maintain the hyperammoniemia at about 10 μg per milliliter. No clinical sign of damage was apparent. The resin was then suspended for 3 weeks while the animals were fed a normal Purina Chow diet. Finally, the protective dose of arginine, followed one hour later by the intraperitoneal lethal dose of ammonium acetate, was injected, and all animals survived.

A chronic hyperammoniemia was able to nullify the protective action of arginine toward rats challenged with the intraperitoneal lethal dose of ammonium acetate. The protective action of arginine decreased more rapidly with higher levels of ammoniemia and reappeared when the hyperammoniemia was discontinued. It seemed that free blood ammonia was destroying some component

*Ten times the physiologic level.

of the liver which could be restored on the cessation of hyperammoniemia. On this hypothesis, experiments were carried out in which the protective dose of arginine was supplemented with a liver homogenate.

Arginine Supplemented With Liver Homogenate.—It was found that 50 per cent of rats receiving 15.6 mM. per kilogram of ammonium acetate subcutaneously died in about one hour. However, if one hour before poisoning each animal received subcutaneously 0.5 Gm. of rat liver homogenized in 2.0 ml. of a solution containing 2.0 mM. per kilogram L-arginine · HCl, 2.0 mM. per kilogram D-glucose, and 1.5 mM. per kilogram sodium chloride, complete protection was achieved. As shown in Table I, arginine alone, no matter how large the dose, was unable to protect rats poisoned by the subcutaneous doses of ammonium acetate. Complete survival was obtained even if the mixture with the homogenate, kept at room temperature for 15 to 30 minutes, was injected simultaneously with the toxic dose of ammonium acetate. However, omission of one of the components or boiling of the complete mixture destroyed the protective action.

TABLE II. TOTAL PHOSPHORUS AND POTASSIUM IN LIVER PARENCHYMA OF RATS POISONED WITH AMMONIUM ACETATE OR PROTECTED WITH L-ARGININE · HCl

	BEFORE TREATMENT	AFTER $LD_{99.9}$ IP AMMONIUM ACETATE*	AFTER $LD_{99.9}$ SUBCUTANEOUS AMMONIUM ACETATE†	60 MIN. AFTER SUBCUTANEOUS LD_{50} AMMONIUM ACETATE‡	60 MIN. AFTER IP $LD_{99.9}$ AMMONIUM ACETATE§
Total phosphorus	411 ± 25	348 ± 20	312 ± 15	410 ± 20	409 ± 15
Potassium	380 ± 20	300 ± 16	295 ± 23	376 ± 21	370 ± 21

Numbers represent milligrams per 100 Gm. of fresh tissue. Averages of duplicate determinations on groups of 10 rats.

*10.8 mM. per kilogram.

†37.0 mM. per kilogram.

‡0.5 Gm. rat liver in 2 ml. water + 2 mM. per kilogram L-arginine · HCl + 2 mM. per kilogram D-glucose + 1.5 mM. per kilogram NaCl prior to 15.6 mM. per kilogram of ammonium acetate subcutaneously.

§4 mM. per kilogram L-arginine · HCl one hour prior to 10.8 mM. per kilogram ammonium acetate intraperitoneally (IP).

In another experiment, the change in total phosphorus and potassium ions in the liver was used as a criterion on which estimation could be made of the degree of liver damage in two groups of rats poisoned by the intraperitoneal or the subcutaneous route. Table II shows that the level of both ions in each group decreased about 20 per cent after the injection of the ammonia donor. If the animals were protected against ammonia intoxication, phosphorus and potassium returned rapidly to normal levels and no appreciable difference was found between the two groups. However, although the intraperitoneally poisoned rats survived with a simple injection of arginine, the subcutaneously poisoned rats needed arginine supplemented with liver homogenate, glucose, and sodium chloride. It would appear that the liver homogenate is supplying or restoring some factor necessary for the utilization of arginine. In the following experiments, in which the level of arginine in the peripheral blood is correlated with the percentage of survivors, further evidence is obtained in support of such a supposition.

Free Arginine in Hepatic Blood.—Two groups of rats received 4 mM. per kilogram of L-arginine · HCl intraperitoneally. At the same time interval, the levels of free arginine in hepatic blood were determined in one group, while the second group was challenged with the intraperitoneal $LD_{99.9}$ of ammonium acetate. The same experiment was repeated, with the injection of 8 mM. per kilogram L-arginine · HCl as a protective dose. Although free arginine in the hepatic blood reached a maximum in a few minutes after the intraperitoneal injection, and then decreased, the percentage of survivors followed an opposite pattern. Table III shows that a high concentration of arginine in hepatic blood at the time of administration of a toxic dose of ammonium acetate does not necessarily correlate with a high survival rate.

In the second experiment, 3 groups of 20 rats each were injected with 1.0 mM. per kilogram of arginine directly into the spleen and were then challenged

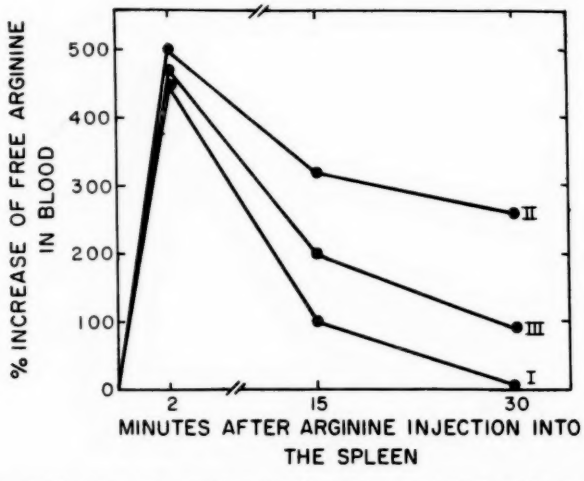

Fig. 4.—Utilization of arginine by livers of protected and unprotected rats. Free arginine in hepatic blood: I = 1.0 mM. per kilogram L-arginine · HCl into the spleen + $LD_{99.9}$ ammonium acetate intraperitoneally (100 per cent survivors). II = 1.0 mM. per kilogram L-arginine · HCl into the spleen + $LD_{99.9}$ ammonium acetate subcutaneously (no survivors). III = 1.0 mM. per kilogram L-arginine · HCl into the spleen + 1.0 ml. saline.

TABLE III. RELATIONSHIP BETWEEN AMOUNTS OF ARGININE IN THE BLOOD DRAWING FROM THE LIVER AND PER CENT SURVIVORS AFTER INTRAPERITONEAL LETHAL DOSE ($LD_{99.9}$) OF AMMONIUM ACETATE

	L-ARGININE INJECTED INTRAPERITONEALLY			
	4 mM. PER Kg.		8 mM. PER Kg.	
TIME OF BLOOD SAMPLING* (MINUTES)	ARGININE IN HEPATIC BLOOD (γ/ML.)†	PER CENT OF SURVIVORS‡	ARGININE IN HEPATIC BLOOD (γ/ML.)†	PER CENT OF SURVIVORS‡
0	7		6	
2	39	20	45	20
15	30	40	62	40
30	29	50	58	40
60	18	100	27	100

*At zero time the blood was withdrawn from the hepatic vein before any treatment.

†Each number represents an average on 5 rats. The values agreed ± 20 per cent.

‡Numbers indicate the percentage of surviving rats when L-arginine · HCl in quantity of 4 or 8 mM. per kilogram and $LD_{99.9}$ of ammonium acetate were injected intraperitoneally at the time indicated in the first column.

immediately with a lethal dose of ammonium acetate as follows: Group I received the intraperitoneal $LD_{99.9}$ (10.8 mM. per kilogram), Group II, the subcutaneous $LD_{99.9}$ (37.0 mM. per kilogram), and Group III received 1 ml. saline intraperitoneally. The average free arginine levels in the hepatic blood of these 3 experimental groups at 2, 15, and 30 minutes following the injection of ammonium acetate are presented in Fig. 4. It can be seen that 30 minutes after poisoning the free arginine content of the subcutaneously injected rats (Group II) was 3 times higher than that of the intraperitoneally injected animals (Group I). In spite of this high level of arginine all the rats of Group II died while the animals of Group I survived. Delayed disappearance of the arginine from the blood of animals in Group II may indicate that the liver had a decreased capability to utilize arginine. Furthermore, as Fig. 4 shows, the disappearance of the arginine from the hepatic blood of normal rats (Group III) is slower than in protected animals (Group I).

TABLE IV. UREA EXCRETED BY PARTIALLY HEPATECTOMIZED RATS WITH HIGH BLOOD AMMONIA

ANIMALS	INGESTED DIETARY N (MG. PER DAY)	NH_3 FED AS DOWEX–50 (MG. PER DAY)	UREA N (MG. PER DAY)*	$NH_3 \cdot N$ (MG. PER DAY)*
Group I†	252	150	280	16
Group II	254	150	240	15
Group III	256	0	130	11
Group IV	250	0	122	9

*Numbers represent averages on 4 days' excretion. The difference in urea excretion between Groups I and II was statistically significant at the probability level of 0.01. The difference between Groups III and IV was not statistically significant.

†Each group represents 10 rats on arginine-free diet. Group I receiving daily 2.0 mM. L-arginine · NCl + 32.0 mEq. ammonia as Dowex–50 + 32.0 mEq. sodium bicarbonate. Group II receiving daily only 32.0 mEq. ammonia as Dowex–50 plus 32.0 mEq. sodium bicarbonate. Group III receiving daily only 2.0 mM. per kilogram L-arginine. Group IV receiving daily only 2.0 ml. water.

Influence of Arginine on Urea Production (Table IV).—It has been shown already that blood urea was increased in animals protected by arginine against acute ammonia poisoning.[3] The following studies examined the ability of arginine to augment the urea-forming capacity of a liver chronically overloaded with ammonia. It has been assumed that a rat liver was "chronically overloaded" when a continuous blood ammonia level was kept so high that a further slight increase would kill the animal.

A chronic high level of ammonia could be induced and maintained by force feeding of an ammonia donor resin (Dowex–50). In a normal rat, sufficient resin to "overload" the liver could not be administered; however, this could be achieved in a partially hepatectomized animal.

In a preliminary experiment it was ascertained that about 2.0 mM. per kilogram per day could be injected intraperitoneally for 3 weeks in hepatectomized rats without appreciably disturbing urea excretion, and that rats deprived of about 70 per cent of their liver were killed by a quantity of resin containing more than 140 mg. of ammonia.

In subsequent experiments, 4 groups of 10 rats each were kept on an arginine-free diet 7 days before removal of about 70 per cent of their liver.

The first day after the operation all animals received water only, ad libitum. On the second day, each animal received 15 ml. of an arginine-free diet; on the third, fourth, and fifth day each received 22.0 ml. The animals consumed all the diet given, so each group had a comparable nitrogen intake.

Group I, from the second to the fifth day after hepatectomy, received a daily intraperitoneal dose of 2.0 mM. per kilogram L-arginine · HCl plus 32.5 mEq. per kilogram sodium bicarbonate. The mixture was divided into 4 doses and injected in 0.5 ml. water solution at 4 hour intervals. In addition, Dowex–50 × 8, in ammonia cycle, corresponding to 16.0 mEq. per kilogram of ammonia, was given twice daily by stomach tube at 8 hour intervals. Group II received the same treatment as Group I except that the arginine injection was omitted. Group III was injected with only 0.5 ml. water 4 times a day.

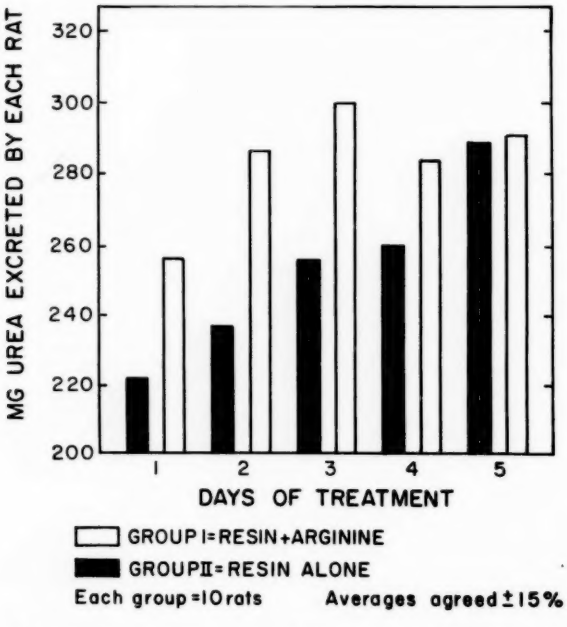

Fig. 5.—Average quantity of urea excreted per day by partially hepatectomized rats during chronic ammonia poisoning.

Table IV summarizes the results.* Under conditions of equal nitrogen intake Group I, receiving arginine, excreted more urea than Group II. Fig. 5 shows the daily differences in amount of urea excreted by rats of Groups I and II. The highest values were obtained on the second and third days from the beginning of the treatment and ranged between 45 and 50 mg. of urea per animal per day. The quantity of urine excreted was consistent with the increased urea production, being greater in Group I than in Group II. It seems clear that arginine can increase the quantity of urea excreted by partially hepatectomized rats chronically poisoned with a quantity of ammonia close to

*We wish to thank J. Lieberman (National Institutes of Health, Division of Research Service) for statistical evaluation of the data.

the lethal dose. Of course, the regeneration of the liver is very fast and, therefore, this effect can be observed only for the first 5 days following hepatectomy.

DISCUSSION

The first demonstration that arginine could prevent ammonia intoxication was given in 1955.[1] Since then the clinical experience obtained by administering arginine to patients with liver diseases raised the question whether a damaged liver could utilize arginine. The experimental data reported here show: (a) Chronic hyperammoniemia in some way injures the liver so that the arginine protective action is nullified. (b) A liver damaged by ammonia utilizes less injected arginine than a normal liver. (c) A protective action of arginine can be restored by the injection of liver homogenates. (d) Under certain conditions arginine can increase the urea-forming capacity of the liver. Two of these results should be emphasized: (1) A chronic excess of free blood ammonia, although small, can greatly impair the liver functions and, therefore, the duration of hyperammoniemia is a critical factor. (2) The injection of liver homogenates restores the protective action of arginine after it has been nullified by the chronic hyperammoniemia. It is probable that some component of the system which utilizes arginine for the production of urea is restored by the homogenate.

SUMMARY

Studies were undertaken to assess the role that the liver plays during the protective action of arginine against ammonia intoxication. It has been shown that:

1. Livers of protected rats utilized arginine faster than livers of unprotected animals and even faster than those of normal rats.

2. Partially hepatectomized rats bearing high levels of blood ammonia increased the over-all excretion of urea when treated with small doses of arginine.

3. The protective action of arginine could not be directly correlated with the amount of free arginine in the hepatic blood or with the levels of ammoniemia.

4. A low but chronic hyperammoniemia nullifies the protective action of arginine against acute ammonia toxicity, whereas, during acute poisoning, arginine is effective at levels of blood ammonia 100 times higher than the normal and 10 times the chronic level. Restoration of the protective action of arginine during chronic hyperammoniemia has been achieved by the injection of liver homogenates. This has been interpreted as evidence for a specific damage by free ammonia itself to some liver system involved in ammonia metabolism. This damage depends not only on the absolute level of the ammoniemia but also on the length of time that free ammonia is present in the blood.

The skillful assistance of Flora H. Grantham is gratefully acknowledged.

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EXPERIMENTAL STUDIES ON THERAPY OF CIRCULATORY FAILURE PRODUCED BY ENDOTOXIN

MAX H. WEIL, M.D.,* AND BRUCE S. MILLER, B.S.**
LOS ANGELES AND DUARTE, CALIF.

A vasopressor agent, adrenergic blocking agent, sympatholytic drug, and a corticosteroid hormone were used in the treatment of shock produced by the injection of Echerichia coli endotoxin into adult dogs. The therapeutic agent was administered fifteen minutes after shock had occurred.

Single intraveous injections of Dibenzyline, phentolamine, and prednisolone were employed. Metaraminol was administered by intravenous infusion to maintain mean arterial pressure at a level 20 mm. less than control value, for a period of two hours. In each experiment, one animal was treated and the other served as a simultaneous control. A total of eighty-two dogs was studied. Arterial and venous pressure, heart rate, urine flow, hematocrit, pH, serum transaminase, survival, and pathologic changes in the nonsurvivors were observed.

The vasopressor agent and corticosteroid hormones increased the period of survival and the number of survivors. Adrenergic blocking agent and sympatholytic drug decreased the survival period. Combined use of the vasopressor agent and corticosteroid provided the longest survival periods and minimized injury, as was reflected in lesser elevation of serum transaminase, less blood pH reduction, and maintenance of almost normal output of urine.

Pretreatment with corticosteroids or with adrenergic blocking drugs has been shown by others to protect against lethal effects of endotoxin. In the present experiments, corticosteroid was also effective for treatment of endotoxic shock. However, adrenergic blocking agent and sympatholytic drug were not beneficial after the onset of shock, whereas the judicious use of a sympathomimetic drug as a vasopressor agent did not promote irreversibility of the shock state, but prolonged the survival period.

HYPOTENSION and shock occurring in patients during the course of bacteremia due to Gram-negative bacteria has been an increasingly serious problem for the clinician.[1, 2] The mechanism of this type of shock is not fully understood. It is generally accepted that shock is caused by the endotoxin which is released from the Gram-negative bacteria. Endotoxins from various enteric bacteria constitute a group of chemically similar substances, lipopolysaccharides, which are an integral part of the viable bacterial cell. During

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disintegration of the bacterial cell, which may be spontaneous or induced by antibiotics, the endotoxin is released to trigger a series of characteristic pathophysiologic reactions, including hypotension and shock.[3-5]

Vasopressor agents minimize splanchnic pooling and thereby increase venous return, an action which opposes the initial hemodynamic disturbances which follow intravenous injection of endotoxin in dogs.[6] However, the possibility clearly exists that severe arteriolar spasm and capillary injury, which are prominent during the later stages of the shock reaction, are actually augmented by this group of drugs. Under these circumstances, epinephrine, norepinephrine, metaraminol, and other sympathomimetic amines might be detrimental, and drugs which have sympatholytic or adrenergic-blocking actions would be more likely to increase chances of survival.

The effects of vasopressor agents, adrenergic blocking agents, and corticosteroids during shock produced by endotoxin were recently evaluated by Lillehei and MacLean.[7] In their studies on dogs, vasopressor agents were administered before or coincidentally with endotoxin, thereby preventing the blood pressure fall caused by the bacterial toxin. Dibenzyline, chlorpromazine, and hydrocortisone were administered ½ to 5 days prior to shock production. Under conditions of these experiments vasopressor agents, particularly metaraminol, hastened a fatal outcome, whereas pretreatment with adrenergic blocking drugs and corticosteroid hormones protected against the deleterious effects of endotoxin.

The purpose of the present experiments was to study the therapeutic rather than the protective effects of vasopressor amine, sympatholytic drug, adrenergic blocking agent, and corticosteroid during endotoxic shock. Each of the drugs was administered only after shock had occurred.

MATERIALS AND METHODS

Studies were made in mongrel adult dogs of both sexes. Sodium pentobarbital was administered intravenously in an amount of 30 mg. per kilogram body weight, with supplementary doses as needed for anesthesia. An endotracheal tube was inserted and atropine was used in a dosage of 0.4 mg., repeated at ½ to 3 hour intervals to minimize tracheobronchial secretions and to block excessive vagotonic effects of the vasopressor agent. Previous observations indicated that this did not materially alter the progress of the shock state.[6] Experiments covered an 8 month period during which there were uncontrolled variations in climatic conditions. For this reason each experiment was separately controlled. Two dogs, usually of the same sex and of comparable age and weight, were used. Both dogs were treated in an identical manner, except that only one received the therapeutic agent under study. The other animal received inert diluent. The experimental animal was selected by the flip of a coin just prior to the injection of endotoxin.

Arterial pressures were measured in the aorta through a catheter advanced from the femoral artery, and venous pressure was measured in the inferior vena cava through a catheter inserted through the femoral vein. Strain gauge pressure transducers (Statham 23AA) and a direct-writing Sanborn multichannel recorder were employed. Mean pressures were obtained by electrical integration. Patency of the catheters was maintained by intermittent flushing with heparinized isotonic saline or Ringer's solution under pressure, or by a slow intravenous infusion of 5 per cent glucose solution. Between 10 and 25 ml. per kilogram of fluid were administered during a 5 hour recording period.

Arterial pressure, venous pressure, and heart rate were measured at 15 minute intervals. An indwelling catheter was maintained in the bladder of male dogs and urine output was measured at 30 minute intervals. Measurements of hematocrit, pH, and serum glutamic oxaloacetic transaminase (SGOT) were made on arterial samples obtained during the control period and at 1 hour intervals after injection of endotoxin. The Wintrobe method was used for hematocrit determination and the SGOT was measured according to the method of Karmen and associates.[8] Blood pH was recorded on a Varian strip chart recorder, using a continuous blood flow electrode and a Beckman "W" pH meter designed to measure between 6.0 and 8.0 pH units. Calibrations were made at pH 6.8, 7.2, and 7.6, and the recorded value was interpolated, providing an accuracy of ±0.01 pH unit of the buffer value.

TABLE I. THE EFFECT OF THERAPY ON TIME OF DEATH AND SURVIVAL FOLLOWING PRODUCTION OF SHOCK WITH ENDOTOXIN

THERAPY	NUMBER		ENDOTOXIN (MG./KG.)	TIME OF DEATH* (HOURS)		SURVIVORS†		STATISTICAL SIGNIFICANCE
	TREATED	UNTREATED		TREATED	UNTREATED	TREATED	UNTREATED	
A. Metaraminol	9	9	3.6	13.6 ± 3.5	10.9 ± 1.9	0	0	
B. Prednisolone	9	9	6.6	13.0 ± 3.8	6.6 ± 1.6	3	2	$p = .02$
C. Prednisolone and metaraminol	9	9	3.5	25.1 ± 4.9	14.1 ± 4.6	4	2	$p = .01$
D. Dibenzyline	5	5	4.2	4.8 ± 2.2	11.1 ± 3.3	0	0	$p = .05$
E. Phentolamine	5	5	3.1	6.8 ± 2.8	8.5 ± 1.5	1	2	
F. Control		8	None				8	

*Arithmetic mean and standard deviation.
†Number of dogs which survived for 48 hours or more.

A preparation of crude endotoxin was used which consisted of dried bacterial cells made from *E. coli* grown on synthetic, protein-free media according to methods previously described.[9] A single intravenous injection was used to produce a shock estimated to be lethal in 90 per cent of control animals. The amount of endotoxin required to obtain this effect varied with different lots of endotoxin and ranged from 3.8 to 7.5 mg. per kilogram body weight. Treatment was begun 10 and 15 minutes after the injection of endotoxin. The drugs were injected or infused into the femoral vein according to the following protocols:

A. Metaraminol (Nine Experiments).*—A solution containing 50 mg. of metaraminol bitartrate per liter of 5 per cent glucose in water was infused into the femoral vein for a period of 2 hours. Rate of infusion was adjusted so that mean arterial pressure was maintained at a level which was 20 mm. Hg less than was recorded prior to injection of endotoxin.

B. Prednisolone† (Nine Experiments).—Prednisolone phosphate was administered as a single intravenous injection in an amount of 20 mg. per kilogram body weight.

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RESULTS

Survival.—Treatment with metaraminol, prednisolone, and a combination of these drugs prolonged the survival period; following prednisolone, or the combination of the drugs, the number of survivors of shock caused by endotoxin was increased. Dibenzyline and phentolamine shortened the survival period. Time of death, with and without treatment, and statistical analyses of the differences are shown in Table I.

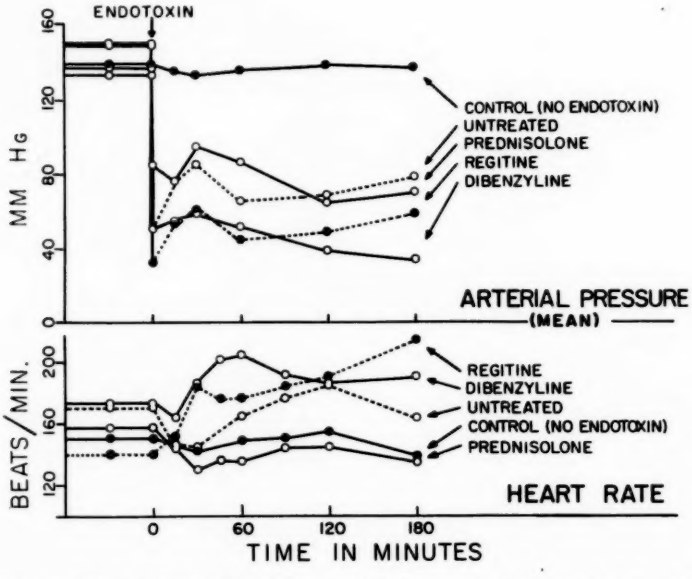

Fig. 1.—Average values of arterial pressure and heart rate in dogs during endotoxin shock following treatment with adrenergic blocking drugs and corticosteroids.

Arterial Pressure.—The arterial pressure values of prednisolone-treated and untreated animals were similar. Following administration of dibenzyline or phentolamine, the mean arterial pressure was maintained at a level which was approximately 30 mm. Hg less than the level of the controls. These features are demonstrated in Fig. 1. No difficulty was encountered initially in maintaining arterial pressure in the desired range with the metaraminol solution. After the first hour of treatment the arterial pressure remained at this level in most of the animals so that no additional medication was required during the second hour.

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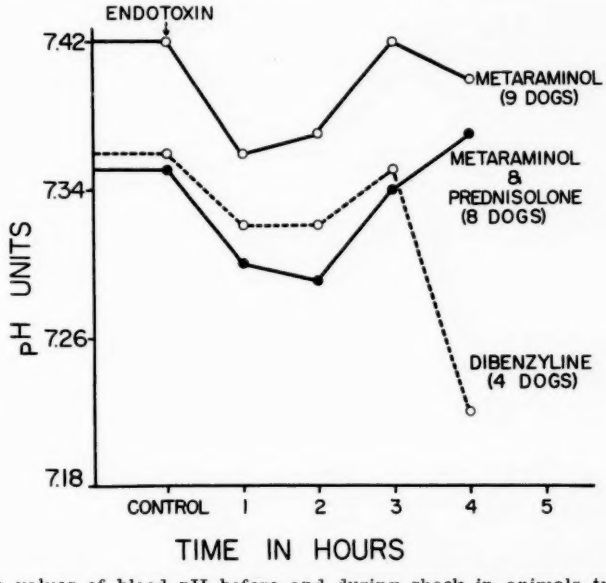

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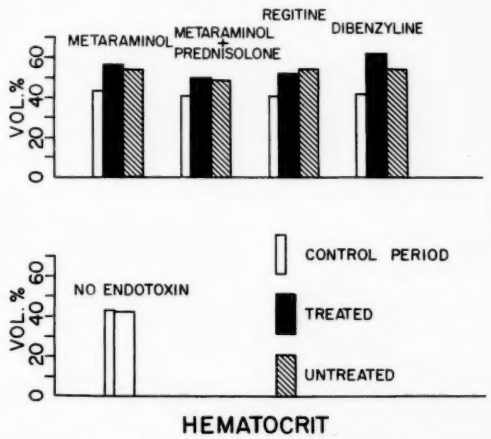

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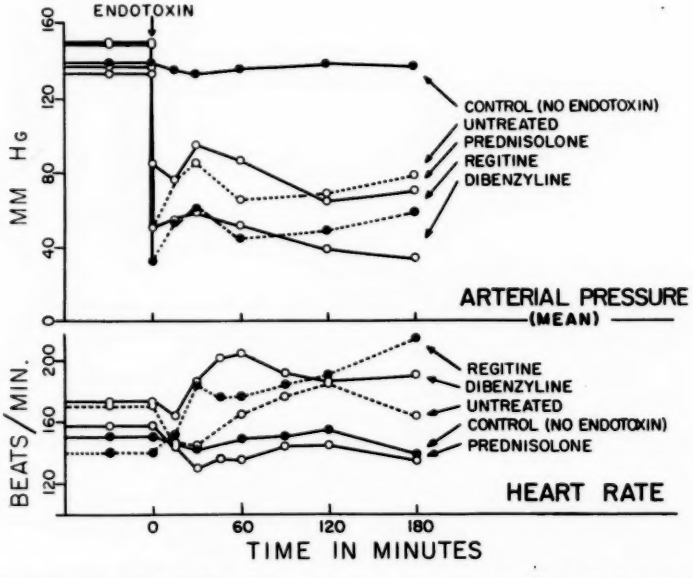

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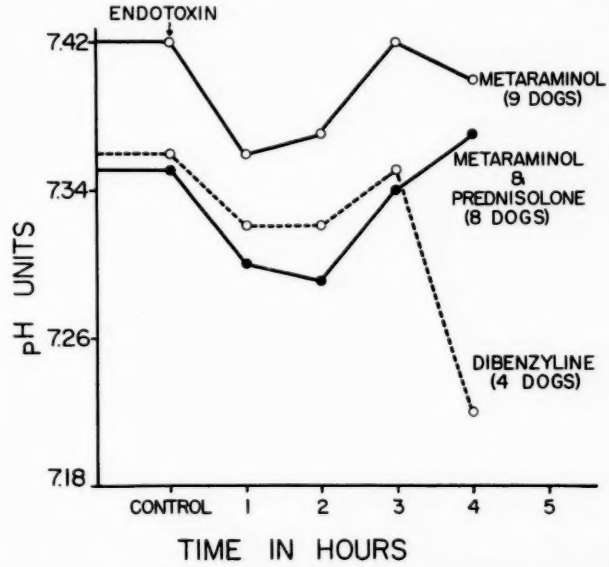

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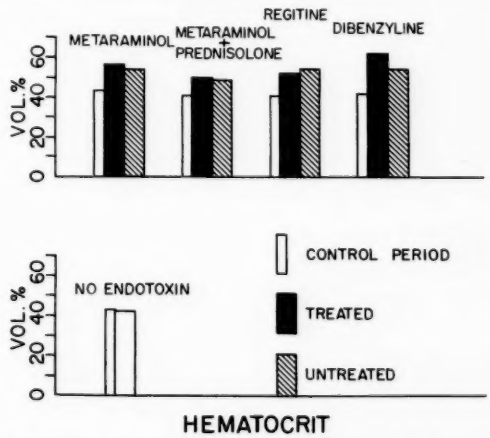

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prednisolone demonstrated an increase in pH between the first and fourth hours. However, following treatment with Dibenzyline and phentolamine, there was a further decline in pH during this period (Fig. 2).

Hematocrit.—Shock produced by endotoxin is associated with progressive hemoconcentration because of a selective loss of plasma from the intravascular space.[10] This reduction in plasma volume is reflected in a progressive increase in hematocrit. In these experiments the extent to which the hematocrit was increased during the 4 hours that followed injection of endotoxin was not clearly related to survival. Therapy did not alter the extent of hemoconcentration because no definitive differences between the various treated and the untreated control groups were apparent (Fig. 3).

Serum Transaminase.—Confirmation was obtained of earlier observations by Melby and associates,[11] of the elevation of SGOT titer following administration of endotoxin. A significantly higher elevation occurred in animals receiving metaraminol. The addition of prednisolone prevented the disproportionate elevation observed with metaraminol alone. SGOT levels of animals treated with Dibenzyline, or animals which received a combination of metaraminol and prednisolone, did not differ significantly from the levels of the untreated control animals (Fig. 4).

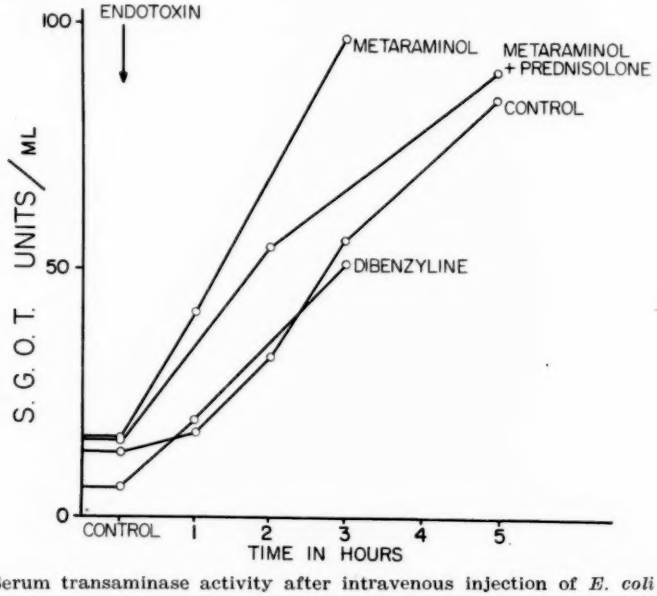

Fig. 4.—Serum transaminase activity after intravenous injection of *E. coli* endotoxin.

Urine Output.—A marked reduction in urine flow followed the onset of shock. Treatment with prednisolone caused slightly lessened decline. However, the combined effect of prednisolone and metaraminol was striking. A majority of these animals maintained urinary flows at levels which sometimes exceeded outputs measured during the control period before injection of endotoxin (Fig. 5).

Pathologic Observations.—Dogs treated with Dibenzyline had slightly less evidence of visceral congestion and submucosal intestinal hemorrhage than the

untreated animals. This was probably related to the rapidity with which these animals succumbed, which decreased the time available for pathologic changes to occur. Dogs which received metaraminol, with and without prednisolone, had no increased evidence of visceral congestion, submucosal edema of the gall bladder or intestine, or intestinal hemorrhage. However, small subepicardial and subendocardial hemorrhages, especially involving the left ventricle, were observed in 7 of 12 dogs which had received metaraminol but in only 2 of 13 control animals.

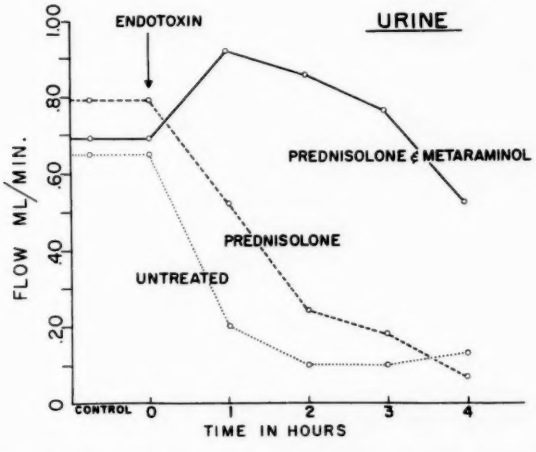

Fig. 5.—Average rates of urine flow in 5 dogs treated with prednisolone, in 5 which received both prednisolone and metaraminol, and in 7 untreated control animals.

COMMENT

Studies on the mechanism of shock caused by endotoxin have provided disparate data that are partially explained by dissimilarity of hemodynamic effects in different animal species. In the dog the immediate response is due to pooling in the portal venous bed proximal to constricted hepatic venules.[12] In the cat, intravenous injection of endotoxin produces immediate constriction of pulmonary venules, leading to acute pulmonary edema and right heart failure.[13] In the monkey and in man, there is usually no immediate reaction and a latent period of 30 minutes precedes a significant fall in blood pressure. Minor degrees of splanchnic as well as pulmonary pooling are detected, but the primary mechanism of shock is not clear.[14-17]

The local effects of endotoxin on the microcirculation were studied by Delaunay and his associates[18] in the mesentery of the guinea pig and the mouse, and in the ear of the rabbit. Tissue injury following intravenous injection of the toxin was ascribed to ischemia resulting from marked constriction of small arteries and arterioles. Algire and associates[19, 20] made their observations on microscopic blood vessels in muscle and connective tissue of mice. They found arteriolar dilatation and venous engorgement at these sites. Zweifach and coworkers[21, 22] made microscopic observations in the mesoappendix of the rat and concluded that the primary site of injury was the capillary blood vessels. In

the dog, the splanchnic veins and venules are distended with blood, whereas the amount of blood in the veins in skin and muscle is reduced during shock caused by endotoxin.[12]

These differences demonstrate the variability of hemodynamic responses to endotoxin, not only from one species to another, but also among various vascular beds within the same species. If therapeutic implications are derived from observations of a selective action of endotoxin at a specific organ or tissue site in a specific species, their applicability to other tissues and different species cannot be assumed. Because it has been assumed that some of these isolated observations have general validity, the major damaging effects of endotoxin are attributed to arteriolar spasm and capillary ischemia. These assumptions have led to controversy over the use of vasopressor drugs during shock caused by endotoxin. Detrimental effects of sympathomimetic drugs were emphasized by Zweifach, Nagler, and Thomas[21] when they reported that, in the rat mesoappendix and rabbit ear, epinephrine and norepinephrine potentiated the local vasospastic response caused by endotoxin. They believed that these compounds, whether endogenous or injected, were largely responsible for the ischemic injury. This point was examined specifically in other tissues and another species by Hinshaw and co-workers,[23] who were not able to show increased sensitivity to epinephrine after endotoxin in the isolated perfused forelimb and lung of the dog. Meyer and Ballin[24] subsequently repeated the studies of Zweifach, Nagler, and Thomas on the isolated, perfused rabbit ear and also were unable to demonstrate potentiation of epinephrine activity. In an investigation by Chedid and Boyer[25] deaths resulting from the systemic effects of endotoxin in the mouse were not increased but reduced by treatment with epinephrine. It is concluded that the evidence implicating epinephrine in causation or potentiation of this type of shock is insecure. Nevertheless, chemicals which block sympathetic nervous activity, or oppose the effect of sympathomimetic drugs, protect against the local vascular damage caused by endotoxin. Pretreatment with Dibenzyline protects rabbits and mice against the systemic lethal effects of endotoxin.[26, 27] However, Abernathy[28] was unable to show that Dibenzyline was effective in increasing survival of mice *after* endotoxin had been injected. Lillehei and MacLean[7] found that pretreatment of dogs with adrenergic blocking drugs protected against fatal progression of shock, and that preshock injection of large amounts of vasopressor agents promoted irreversibility of hypotension. The experiments reported herein showed that adrenergic blocking or sympatholytic drug had no advantage after shock had been produced. On the other hand, treatment with Dibenzyline and phentolamine resulted in earlier death; with moderate doses of a vasopressor agent chances of survival were improved, although the therapeutic value of metaraminol was not a decisive one. The possibility that metaraminol decreases survival can be statistically disproved ($p = 0.01$).

Pretreatment with corticosteroid hormones is remarkably effective in preventing the lethal course of endotoxin shock in the mouse,[29, 30] rabbit,[31] rat,[32]

and dog.[7] The present experiments prove that in the dog it is also *therapeutically* effective when used early and in large doses. Best results were obtained when both corticosteroid and the vasopressor agent were used.

Arterial pressure admittedly is of limited significance in estimating the severity and the prognosis of shock. Although corticosteroids were effective in the treatment of endotoxin shock, their use was not accompanied by significant elevation of blood pressure. However, it seems reasonable to assume that the additional reduction in arterial pressure and increased heart rate that followed treatment with Dibenzyline and phentolamine may provide unfavorable conditions during prolonged shock. This postulation is supported by observations that reduced coronary blood flow and myocardial failure may be important factors contributing to the fatal outcome in both the dog and human patients.[2, 12]

The fall in blood pH reflects inadequate tissue perfusion and accumulation of acid metabolites. Additional studies showed that this is related to a reduction of oxygen consumption, increase in blood lactic acid concentration, and a sharp fall in pCO_2.[33] After maximal buffer capacity of blood is exceeded, the blood pH falls. Administration of alkalinizing drugs, such as sodium lactate or sodium bicarbonate, to maintain pH near "normal" values failed to promote survival and hastened a fatal outcome in 6 treated animals when compared with their untreated controls.

The extent to which plasma is lost from the circulation does not appear to be a major factor in determining the outcome. Therapy which prolonged survival did not result in lowering of the hematocrit, nor does the use of plasma or plasma substitutes prolong survival.[7]

A vasopressor agent may have potentially injurious effects, two of which were demonstrated in the present studies. Serum transaminase, a sensitive but not a specific index of cellular injury, promptly increases during shock produced by endotoxin.[11] The increase was further accentuated after treatment with metaraminol. The possibility exists that the vasopressor agent did not actually increase tissue injury, but mobilized the enzyme from inactive pools. However, this seems less likely in view of increased frequency of subepicardial and subendocardial petechiae on autopsy examination of dogs treated with metaraminol. Others have shown that administration of vasopressor agents, such as metaraminol, may cause ischemic necrosis in myocardium and other tissues and particularly in the liver,[34] and this may account for the transaminase increase observed in the present experiments. It is clear that epinephrine and related vasopressor amines are detrimental when used in excessive quantities, whether in the normotensive or hypotensive animal. However, the data obtained in experiments reported herein indicate that the conservative use of vasopressor agent to support arterial pressure does not increase severity of shock in dogs but instead increases survival.

A marked reduction in urine flow occurs after injection of endotoxin. Urine production is only slightly increased by treatment with prednisolone alone, but essentially normal urine production is established when a combination of prednisolone and metaraminol is used for treatment. The combination

may have another advantage. As already stated, serum transaminase was higher in dogs with endotoxic shock treated with metaraminol, and it was believed that metaraminol itself had caused this increase. When corticosteroid and metaraminol were used together the transaminase was not significantly different from that in the untreated dogs. The corticosteroid presumably protects against the endotoxin. It may also protect against possible detrimental effects of the vasopressor agent, as suggested by Lillehei and MacLean.[35]

The pharmacologic mechanisms that account for the differences between prophylactic and therapeutic effects of the drugs that reduce the action of vasopressor amines are not clear. Pretreatment presumably alters the state of vascular beds, making them a less susceptible target for the endotoxin. However, similar conditions do not obtain after endotoxin has triggered the shock reaction, present studies showing that adrenergic blocking agents and sympatholytic drugs are of no value for treatment and are probably detrimental. The same considerations are pertinent to the use of vasopressor agent although the circumstances are reversed. Pretreatment with vasopressor agent produces alterations in the state of the vascular bed, making it much more susceptible to the damaging effects of endotoxin.[7, 35] However, the cautious use of vasopressor agents during shock, after arterial pressure is markedly reduced, delays rather than promotes the onset of irreversibility in dogs.

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RENAL HEMODYNAMIC CHANGES IN PULMONARY EMPHYSEMA

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The role of cardiac insufficiency and concomitant renal hemodynamic abnormalities in chronic pulmonary insufficiency has not been clearly defined. For this reason renal clearances, cardiac outputs, and pulmonary pressures were measured before and after isoproterenol infusions in seventeen cases which met clinical and laboratory diagnostic criteria for severe pulmonary emphysema.

Glomerular filtration rates and renal plasma flows were determined by measurement of urinary clearances of infused inulin and para-aminohippurate. Cardiac catheterization was performed concurrently, facilitating simultaneous measurement of cardiopulmonary pressures and cardiac outputs.

Ten patients without past or present cardiac decompensation exhibited mean values of 3.04 L. per minute per square meter for the cardiac index, 389.8 ml. per minute for renal plasma flow, and 27.2 for the filtration fraction, compared to means of 2.93 L. per minute per square meter, 505.3 ml. per minute, and 21, respectively, for a comparable control group. During isoproterenol infusions mean values changed to 4.55 L. per minute per square meter for the cardiac index, 450 ml. per minute for renal plasma flow, and 23.4 for the filtration fraction. Seven patients with recent congestive heart failure averaged 2.45 L. per minute per square meter for the cardiac index, 279.7 ml. per minute for renal plasma flow, and 31.7 for the filtration fraction. During isoproterenol infusions mean values changed to 4.57 L. per minute per square meter for the cardiac index, 317.4 ml. per minute for renal plasma flow, and to 23.4 for the filtration fraction.

The results indicate that renal plasma flow can fall significantly in pulmonary insufficiency prior to the onset of overt cardiac decompensation. The close relationship of renal plasma flow to cardiac output is emphasized by the significant rise in both functions during isoproterenol infusions. It is suggested that relative cardiac insufficiency and concomitant renal hemodynamic abnormalities may precede and initiate other manifestations of pulmonary heart failure.

IT has become well recognized that congestive heart failure occurs in chronic lung disease, particularly pulmonary fibrosis and emphysema.[1, 2] In general this pulmonary heart failure is believed to be predominantly right ventricular insufficiency resulting from pulmonary hypertension.[3] Arterial anoxemia, polycythemia, hypervolemia, hypercapnia, and increased cardiac output, associated with pulmonary insufficiency, all contribute to cardiac decompensation.

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In pulmonary heart failure, renal plasma flow decreases, and the filtration fraction or ratio of glomerular filtration rate to renal plasma flow increases, as observed in congestive heart failure of nonpulmonary origin.[4, 5] These hemodynamic changes are usually attributed to reduction of the renal fraction of a low cardiac output.[6] Although cardiac output is greater in pulmonary heart failure than in other forms of cardiac decompensation, it should be emphasized that a relatively low systemic blood flow is present as a result of the increased cardiac output requirements peculiar to cor pulmonale.[1] The known major factors responsible for this increased systemic blood flow requirement are increased respiratory muscle oxygen consumption, anoxia, and shunting of blood between the greater and lesser circulation.[7-9]

However, the early contribution of the heart and kidneys to the development of pulmonary heart failure is less well understood. Several authors have reported normal or low normal renal plasma flows prior to the appearance of clinically recognizable right ventricular failure.[4, 10] Stuart-Harris[11] has observed a moderate decrease in renal plasma flow under similar circumstances.

Since the adequacy of the systemic circulation and its relationship to renal clearances seemed of particular importance in the understanding of the early pathophysiologic changes of pulmonary heart failure, this study was designed to correlate cardiac output, right heart pressures, and renal clearances in patients with chronic lung disease. In order to evaluate cardiorenal interdependence, hemodynamic observations were repeated during a pharmacologically induced increase in cardiac output with intravenous isoproterenol.

METHODS

Pulmonary function studies, renal clearances, and right heart catheterizations were accomplished on seventeen randomly selected patients with severe generalized pulmonary emphysema. These patients had been hospitalized primarily because of dyspnea, which had usually been aggravated by infection prior to admission. Ten subjects (Group A) did not have past symptoms or signs suggestive of congestive heart failure, aside from dyspnea. The clinical findings as well as the hospital course tended to confirm the absence of cardiac decompensation and supported the impression of pulmonary dyspnea in each instance. The remaining seven patients (Group B) had past histories of edematous congestive heart failure, and usually exhibited gross signs of cardiac decompensation at the time of hospital admission. Clinical improvement followed anticongestive therapy in this latter group, and all patients were studied only after receiving maximal benefit from treatment.

Pulmonary function was evaluated by determinations of lung volumes, maximal breathing capacity, intrapulmonary gas mixing by the helium open-circuit washout method, and arterial blood gases.

All patients were studied in the hydrated, postabsorptive state and received sedation in the form of either 0.1 Gm. of sodium Amytal or 400 to 800 mg. of meprobamate. Priming intravenous injections of para-aminohippurate and inulin were followed by a continuous infusion at a constant rate calculated to attain optimal blood concentrations approaching 2 mg. per cent for para-aminohippurate and 25 mg. per cent for inulin. Three carefully timed 15 minute urine samples were collected after establishment of constant plasma drug levels. These urine specimens were obtained by gravity and pressure drainage of the bladder through an indwelling retention catheter. Blood samples for inulin and para-aminohippurate analyses were obtained at the beginning of the procedure, at the onset of each urine collection, and at 15 minute intervals thereafter. Clearance values for each period did not vary more than 10 per cent and were expressed as an average for each subject.

During the 45 minutes following the priming injections, a No. 8 double lumen cardiac catheter was passed through an antecubital vein to the pulmonary wedge position. A No. 18 Cournand needle was inserted into a brachial artery. Metycaine was used for analgesic purposes. Intracardiac, pulmonary vascular, and arterial pressures were recorded electronically on a photographic instrument. During the third urine collection, two cardiac outputs were determined sequentially by the Fick method with the use of mixed venous blood from the pulmonary artery. After completion of this initial base-line portion of the procedure, an intravenous infusion of isoproterenol was given at a 1 to 3 meg. per minute rate for one half hour. During this time all initial observations were repeated.

Inulin and para-aminohippurate determinations in blood and urine were performed by standard methods.[12-15] Blood oxygen content and saturation were measured by the photometric method of Hickam and Frayser.[16] Blood carbon dioxide content was determined by the method of Van Slyke and Neill.[17] The pH of whole blood was measured by the Cambridge, Model R, pH meter with enclosed glass electrodes; measurements were corrected to 37° C. with the use of Rosenthal's factor.[18] Plasma carbon dioxide content was calculated from the blood carbon dioxide content, pH, and hemoglobin, with the use of the line chart of Van Slyke and Sendroy.[19] The carbon dioxide tension was calculated from this value, by means of the Henderson-Hasselbalch equation. Oxygen consumption was determined from the volume of expired air and oxygen content as measured in the Pauling oxygen analyzer. All determinations were done in duplicate.

Cardiac indices and renal clearances were compared with values obtained from normal controls of comparable age. Control data from our laboratory were similar to those reported by Davies and Brandfonbrener for various ages.[20, 21]

RESULTS

The results of this study are summarized in Tables I and II. The maximal breathing capacity for the patients without heart failure, Group A, averaged 27.4 L. per minute, compared to 37.9 L. for those with a recent history of congestive heart failure, Group B. The average total vital capacity was decreased to 2.44 L. for Group A, and to 2.85 L. for Group B. The residual volume comprised an abnormally large portion of the total lung capacity, averaging 67.9 per cent for Group A and 57.8 per cent for Group B. In addition, each subject required far longer than a normal 7 minutes to wash out an inspired helium mixture from his lungs. The averages of 20.2 and 17.8 minutes for Groups A and B, respectively, indicated severe impairment of inspired air distribution and mixing. A reduction in arterial oxygen saturation was observed in most cases, with an average of 92.7 per cent for Group A and 85.4 per cent for Group B. Most patients exhibited a significant elevation in the resting arterial carbon dioxide tension, averaging 48.8 mm. Hg in Group A and 53.8 mm. Hg in Group B.

All end diastolic right ventricular, pulmonary wedge, and atrial pressures were normal, with the single exception of one Group B subject with 18 mm. Hg wedge and 12 mm. Hg end diastolic right ventricular pressures. The normal values in Group B are not surprising, since maximal cardiac compensation had been achieved prior to study. Mean pulmonary artery pressures were elevated significantly in four of nine base-line observations in Group A and in six of seven such instances in Group B.

The mean cardiac index in Group A averaged 3.04 L. per minute per square meter and 2.45 L. per minute per square meter in Group B, as compared to a normal control value of 2.93 L. per minute per square meter. Infused isoproterenol increased the cardiac index to 4.55 and 4.57 L. per minute per

square meter for Groups A and B, respectively (Figs. 1 and 2). Systemic arterial pressure was lowered moderately in every instance during the drug infusion.

TABLE 1. PULMONARY PHYSIOLOGIC OBSERVATIONS

PATIENTS	AGE	DIAGNOSIS	MAXIMAL BREATHING CAPACITY BPTS (L./MIN.)	TOTAL VITAL CAPACITY BPTS (L.)	RESIDUAL VOLUME TO TOTAL LUNG CAPACITY (RATIO × 100)	HELIUM WASHOUT TIME (MIN.)	ARTERIAL O_2 SATURATION (%)	ARTERIAL PCO_2 (MM. Hg)
Chronic Pulmonary Disease: No Evidence of Heart Failure at Any Time.—								
H. R.	50	Emphysema	25	2.67	71	15	93	44
A. T.	62	Emphysema	17	1.90	74	31	93	47
S. J.	68	Emphysema, tuberculosis	26	1.60	69	17	92	46
H. S.	38	Emphysema, bronchiectasis	27	2.63	54	30	87	60
P. E.	40	Emphysema	20	1.99	68	30	95	44
L. E.	38	Emphysema	25	2.40	85	25	92	49
R. F.	49	Emphysema	22	1.60	68	16	93	53
S. J.	48	Emphysema	32	2.57	70	11	94	50
B. J.	54	Emphysema	14	1.90	69	18	94	49
B. R.	45	Emphysema	66	4.72	51	10	94	46
Mean			27.4	2.44	67.9	20.2	92.7	48.8
S. D.			14.5	0.91	9.6	8.1	2.2	4.8
Chronic Pulmonary Disease: Clinical Evidence of Heart Failure in the Past.—								
W. W.	54	Emphysema, ASHD*	23	1.80			73	69
F. M.	61	Emphysema, ASHD	22	2.26	69	17	79	59
J. J.	54	Emphysema, ASHD	35	3.69	45	19	92	40
F. T.	42	Emphysema, HCVD†	73	3.73	52	15	92	37
H. J.	61	Emphysema, ASHD	42	3.89	60	17	88	51
W. G.	64	Emphysema, ASHD	49	2.60	60	14	94	51
S. B.	62	Emphysema, ASHD	21	1.98	61	25	80	70
Mean			37.9	2.85	57.8	17.8	85.4	53.8
S. D.			18.9	0.90	8.3	3.9	8.0	13.0
Normal mean values for age and habitus			107	3.87	35	5	93	40-44

*Arteriosclerotic heart disease.
†Hypertensive cardiovascular disease.

The glomerular filtration rate averaged 99.9 ml. per minute in Group A, as compared to 78.7 ml. per minute in Group B. These changes were not significant when compared to normal values for comparable ages. In addition, isoproterenol infusions did not alter significantly the filtration rate in either group.

The effective renal plasma flow in Group A averaged 389.8 ml. per minute, as compared to 279.7 ml. per minute for Group B. These values were significantly lower than the control mean for a comparable age ($P < 0.01$). Infused isoproterenol increased the average flow to 450.4 and 317.4 ml. per minute in Groups A and B, respectively. In Group A the response was statistically significant ($P < 0.02$) (Figs. 1 and 2).

The filtration fraction in Group A averaged 27.2 as compared to 33.5 for Group B; both values were statistically significantly different from normal controls ($P < 0.01$). The filtration fraction fell significantly to 23.4 in Group A during the isoproterenol infusion, and in a smaller number of Group B subjects

TABLE II. CARDIORENAL HEMODYNAMIC OBSERVATIONS BEFORE (b) AND DURING (d) AN INFUSION OF ISOPROTERENOL

PATIENTS	CARDIAC INDEX (L./MIN./M_2)		CENTRAL A. V. DIFFERENCE (VOL. %)		RIGHT VENTRICULAR END DIASTOLIC PRESSURE (MM. Hg)	PULMONARY ARTERY MEAN PRESSURE (MM. Hg)	GLOMERULAR FILTRATION RATE (ML./MIN./ 1.73 M.²)		EFFECTIVE RENAL PLASMA FLOW (ML./MIN./ 1.73 M.²)		FILTRATION FRACTION		RENAL FRACTION	
	b	d	b	d	b	b	b	d	b	d	b	d	b	d
Chronic Pulmonary Disease: No Congestive Heart Failure at Any Time.—														
H. R.	3.34	6.58	4.42	3.11	3	27	120	144	486	650	24.6	22.1	15	11
A. T.	2.23	3.20	5.06	4.22	0	22	137	134	349	389	39.0	34.0	17	15
S. J.	2.54	4.40	4.18	2.99	6	14	81	107	312	480	25.9	22.0	11	10
H. S.	3.33	5.32	4.20	3.30	1	43	95	93	353	401	26.9	23.2	11	8
P. E.	2.12	3.43	5.58	4.26	1	18	96	86	382	489	25.1	17.6	20	16
L. E.	3.25	4.56	4.35	3.38	-1	16	96	95	371	396	25.8	23.9	8	6
R. F.	3.32	5.33	3.85	2.81	4	41	74	78	323	413	23.1	20.8	10	8
S. J.*	4.22		4.11						347				13	
B. J.	2.75	3.56	4.45	3.67	1	16			393	385			16	12
B. R.*	3.26		4.03		0	12			582				17	
Mean	3.04	4.55	4.42	3.54	1.6	23.2	99.9	105.3	389.8†	450.4	27.2†	23.4	13.8	10.8
S. D.	0.63	1.16	0.52	0.54	2.3	11.5	21.8	24.8	83.0	90.3	5.6	5.1	3.8	3.5
Chronic Pulmonary Disease: Clinical Congestive Heart Failure Documented.—														
W. W.*	3.01		4.89		12	48	121		201		60.2		13	
F. M.	2.58	3.81	5.35	3.57	6	30	50	65	178	206	28.0	31.5	6	5
J. J.	2.12	3.96	5.28	3.59	5	22	90	95	385	405	31.5	23.4	15	11
F. T.	2.25	3.37	6.08	5.16	-1	16	45	39	198	208	22.7	18.7	9	6
H. J.*	1.94		5.88		5	38	89		352		25.3		20	
W. G.	3.35	5.27	5.96	3.88	5	27	77	78	340	386	22.7	20.1	9	6
S. B.	1.93	6.46	4.07	2.17		29			404	382			23	7
Mean	2.45	4.57	5.36	3.67	5.3	30.2	78.7	69.3	279.7†	317.4	31.7†	23.4	13.5	7.0
S. D.	0.55	1.27	0.71	1.12	4.1	3.6	28.2	23.6	86.3	101.2	14.3	5.9	6.2	2.3
Normal mean values for age and habitus														
	2.93‡		4.5‡			14‡	105.3§		505.3§		21§		23‡	
							122.4‡		617.2‡		20.2‡			

*Patients could not tolerate length of procedure; last portion of study not accomplished.
†Statistically different from control values $P<0.01$.
‡Normal values for this laboratory.
§Davies and Shock,[20] calculated from data for fifth, sixth, and seventh decades.

the changes were of a similar magnitude and direction (Figs. 1 and 2). The renal fraction was 13.8 and 13.5 per cent for Groups A and B, respectively, and infused isoproterenol decreased these values slightly.

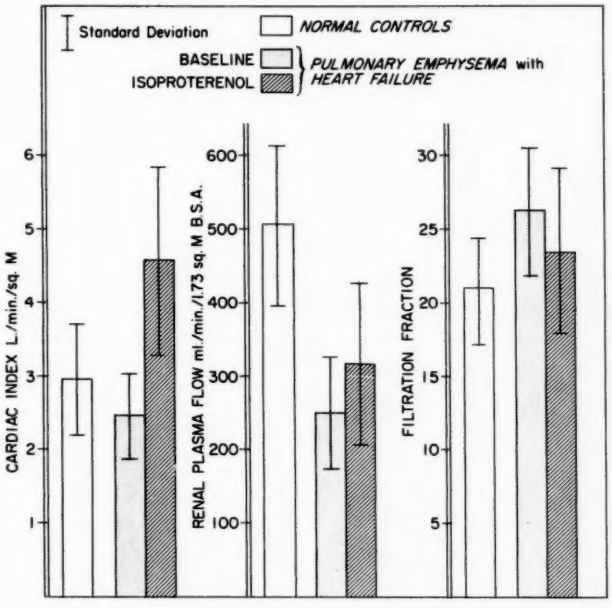

Fig. 1.—Studies on the Group A subjects with emphysema in whom cardiorenal observations were repeated during an infusion of isoproterenol. The graph primarily illustrates the increase in renal plasma flow and decrease in filtration fraction when cardiac output was increased pharmacologically.

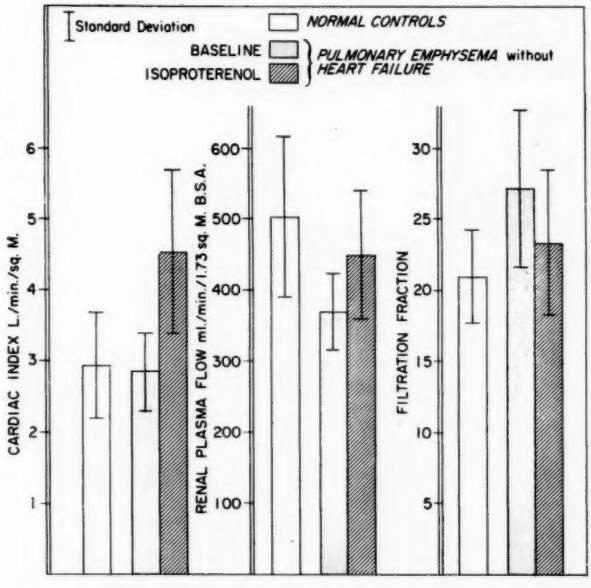

Fig. 2.—Studies on Group B subjects with emphysema and past congestive heart failure in whom cardiorenal observations were repeated during an infusion of isoproterenol. The renal clearance response to an increase in cardiac output was similar to that seen in the Group A patients.

DISCUSSION

The results of this study indicate that renal plasma flow was significantly reduced and that filtration fraction increased concomitantly in the patients with pulmonary emphysema who were studied prior to the development of clinical or hemodynamic evidence of congestive heart failure. The values for renal plasma flow were similar to those reported by Stuart-Harris[11] in a like group of patients.

An inadequate cardiac output seems the most probable mechanism for these renal circulatory changes. The low renal plasma flow and high filtration fraction values of Group A are similar to those reported in low output congestive heart failure.[5, 6] The increase in renal plasma flow and the decrease in filtration fraction when isoproterenol induced a marked rise in cardiac ouput are consistent with the concept of early cardiac insufficiency (Figs. 1 and 2).[22] In addition, the renal fraction was decreased, as is seen in congestive heart failure.[5] The low renal fraction values are particularly significant since cardiac outputs were not initially elevated in this group. The lack of striking changes in the renal fraction during the isoproterenol infusion is also consistent with the possibility that the renal plasma flow increase with isoproterenol relates to a simple rise in cardiac output. Although isoproterenol is active in many parts of the circulation, aside from its strong positive cardiac inotropic effect, it did not increase arterial pressure. Furthermore, sympathomimetic agents have not been demonstrated to increase renal perfusion by direct action.[23] It seems likely, therefore, that the observed increase in renal plasma flow during the drug infusion was not related to a direct effect on the kidney or to a rise in arterial pressure.

A reduced pulmonary blood volume may be present as a result of a contracted pulmonary vascular compartment and could contribute to the development of cardiac decompensation. However, the prompt, large increase in cardiac output observed with isoproterenol infusions suggests that an ample, readily available pool of blood was present in the pulmonary compartment in this group of patients. An early relative reduction in right ventricular output is a more plausible explanation. This change would of necessity restrict left ventricular output. It is not clear how the pulmonary abnormalities might contribute to this process, although pulmonary hypertension does not appear to be the only factor. In this group of patients, coronary insufficiency, anoxemia, and chronic protracted stress may also contribute to cardiac insufficiency.

Aside from cardiac factors, renal vein pressure elevation or humoral agents may be potential causes of decreased renal blood flow and cannot be excluded from consideration on the basis of available evidence, including these studies.[24, 25]

The manner in which decreased renal plasma flow leads to salt and water retention has been studied in other types of heart failure, and, although exact mechanisms are not clear, it is apparent that similar abnormalities of electrolyte and water balance occur in cor pulmonale in association with the development of hydremia and cardiac decompensation.[6, 26, 27]

It is believed that the findings in this study may clarify certain obscure cardiopulmonary renal relationships in heart failure due to pulmonary disease. Clearly pulmonary heart failure can proceed with the same essential sequence of events noted in heart failure of nonpulmonary origin. In an early phase, there is a significant relative reduction in cardiac output. This cardiac insufficiency leads to the classic renal clearance changes noted in other forms of cardiac decompensation which eventuate in sodium and water retention by the body. The further evolution of this process in terms of edema formation and venous hypertension would seem to result from the changes in body fluid dynamics as well as right ventricular disease.

SUMMARY

Renal hemodynamics were evaluated by simultaneous renal clearance and cardiac catheterization studies in pulmonary emphysema patients with and without backgrounds of congestive heart failure. Observations were repeated during an isoproterenol infusion. The principal findings were the statistically significant reductions in renal plasma flow and concomitant increases in filtration fraction for patients with chronic lung disease in whom there were no clinical or cardiopulmonary hemodynamic findings suggesting congestive heart failure.

The close similarity of these renal changes to those reported in cardiac decompensation and the demonstrable improvement in renal clearances when cardiac output rises in Group A are consistent with the suggestion that cardiac insufficiency occurs early in the course of cor pulmonale. This may lead to altered renal hemodynamics and clearances which may precede and contribute to the development of pulmonary heart failure.

Acknowledgment is made of the technical assistance of Mary Rives, Corinna Thomas, and Edward Howard.

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FORMATION OF THROMBOPLASTIC ACTIVITY WITH RUSSELL'S VIPER VENOM

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An incubation mixture of aluminum hydroxide adsorbed plasma, serum, phospholipid, Russell's viper venom, and calcium rapidly produces prothrombin-converting activity. The concentration of the several reactants was varied; the resulting changes in the rate of development and the amount of thromboplastic activity was measured with the use of prothrombin prepared with diethylaminoethyl cellulose anion exchanger. The concentration of serum and adsorbed plasma affected both yield and rate, the concentration of phospholipid affected only the yield. The effect of Russell's viper venom appeared to be enzymatic. The reaction could be divided into two phases; the first involved all factors except phospholipid, the second involved the reaction between the phospholipid and the product of the first reaction. The serum factor is probably Stuart-Prower factor, the plasma factor is probably Factor V. The system described may be adapted for use as an assay for these factors.

THIS paper is concerned with the thromboplastic activity which develops when Russell's viper venom (RVV) is incubated with calcium, a phospholipid, and accessory factors from plasma and serum. Biggs, Douglas, and Macfarlane[1] studied a system in which a purified Factor V preparation and human serum were used as the source of accessory factors, with platelets as the source of lipid, and showed that the incubation of such a mixture with RVV resulted in a pronounced increase in thromboplastic activity. Peden and Peacock[2] studied a similar system in which the accessory factors were provided by beef serum and phosphatidyl ethanolamine isolated from beef brain. The latter report indicated that the first reactions were between the factors in beef serum and RVV, with the phospholipid reacting at some later stage.

In the present work, the accessory factors have been supplied by preparations from human serum and adsorbed human plasma, and the clotting substrate changed from plasma to a mixture of fibrinogen and partially purified prothrombin. These changes have permitted a somewhat clearer picture of the reactions taking place in the incubation mixture, and have permitted more quantitative results than were possible in either of the two systems reported previously. On the basis of the present studies we are able to conclude that the principal time-consuming reactions are those involving the phospholipid, and that some constituent of RVV acts catalytically in the activation of accessory factors in serum and plasma.

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MATERIALS AND METHODS

The buffer used throughout was 0.1 M tris (hydroxymethyl) aminomethane (tris), pH 8.4. Fibrinogen was a 1 per cent solution of Armour Fraction I, in Tris. The phospholipid was the phosphatidyl ethanolamine-rich fraction 5 of Folch,[3] prepared from ox brain and dispersed by mechanical shaking to make a suspension of 1.0 mg. per milliliter in Tris. Dilutions of this stock were prepared as required. $CaCl_2$ 0.025 M was prepared in Tris. Russell's viper venom was the commercial product Stypven. A stock solution of 0.1 mg. per milliliter in the diluent provided was diluted as required in Tris.

Plasma for the preparation of prothrombin was from blood rejected from a blood bank because of a positive serologic test for syphilis. It was separated within 3 days of drawing; in most cases the plasma had been frozen at −20° C. for several weeks before isolation of prothrombin was started.

Prothrombin was prepared by the addition of 1.0 Gm. diethylaminoethyl cellulose* (DEAE) to 50 ml. plasma. The mixture was allowed to stand for about 5 minutes at room temperature and then filtered with gentle suction on a coarse sintered glass funnel of 23 mm. i.d. Before the pad of DEAE became dry, the plasma was poured over the pad of resin for a second adsorption. After the last of the plasma had passed over the DEAE, 20 ml. of 0.85 per cent NaCl was passed over the pad to remove unadsorbed protein. This washing solution was followed by 20 ml. of 0.50 M NaCl, to elute the adsorbed prothrombin. Collection of the sample started with the addition of the 0.50 M NaCl, and suction was continued until the pad was dry. The prothrombin solution was frozen in small portions and diluted 1 : 20 in Tris buffer before use. The prothrombin was stable for several weeks while frozen.

Usually such preparations contained about 250 U. per milliliter, as in the two-stage method of Ware and Seegers,[4] with normal plasma control of 200 U. per milliliter. The average purity was 300 U. per milligram protein (biuret method). These preparations were low in purity but almost devoid of Factor VII, as shown by their failure in correction of the one-stage clotting defect of a patient with congenital deficiency of Factor VII. As a consequence of low Factor VII content, the prothrombin, when tested at 30 U. per milliliter in the prothrombin-proconvertin assay of Owren, as modified by Ware and Stragnell,[5] gave a prolonged clotting time (75 seconds). The clotting time was shortened to 20 seconds when a 1 : 10 dilution of human serum was added. At 30 U. per milliliter there was no Factor V detectable by the method of Lewis and Ware.[6] The prothrombin was able to shorten the one-stage clotting time of plasma from a patient with congenital Stuart Factor deficiency, and the assay described in this paper indicated that Stuart Factor had not been removed. We do not know to what extent other clotting factors may contaminate the prothrombin preparation.

Aluminum hydroxide adsorbed plasma (''alumina plasma'') was prepared from fresh normal citrated plasma by treatment with 1 : 10 volume of alumina gel.[7] Factor V preparations were occasionally made from this material by the method of Lewis and Ware.[8] Such purified preparations and some frozen portions of alumina plasma did not replace fresh alumina plasma in the thromboplastin generations test,[7] and were presumably deficient in antihemophilic globulin (AHG).

Serum used in these experiments was drawn from normal volunteers, allowed to clot for 24 hours at 37° C., and stored at 4° C. for 2 weeks prior to use. Purified serum factor was prepared from serum by the same procedure used for DEAE prothrombin.

The clotting substrate was composed of an equal mixture of 1 per cent fibrinogen and prothrombin solution. The tilt method was used, with a definite web of granules as the end point.

The incubation mixtures (37° C.) were composed of equal volumes of RVV, serum factor, alumina plasma, phospholipid, and $CaCl_2$. A timer was started at the addition of the calcium, which was ordinarily added last, and at suitable intervals 0.1 ml. of the incubation mixture, and an additional 0.1 ml. of calcium were added to 0.1 ml. of the clotting substrate.

*Purchased from the California Foundation for Biochemical Research as the chloride and used without further treatment.

RESULTS

Standard Curve.—When all necessary reagents are present in suitable quantities in the incubation mixture, the mixture is increasingly capable of promoting conversion of prothrombin to thrombin, i.e., it becomes increasingly "thromboplastic." The clotting times, after reaching a minimum, remain constant for a short time before increasing (Table I). A standard curve relating clotting time to thromboplastic activity was prepared by adding to the clotting substrate aliquots of the incubation mixture after it had attained maximum activity.

TABLE I. INCREASE IN THROMBOPLASTIC ACTIVITY OF INCUBATION MIXTURE

Incubation time (min.)	1	2	3	4	5	6	8	10	15
Clotting time (seconds)	32	23	18	17½	18	18½	19	20	22

Two-tenths milliliter each of the following were incubated at 37° C.: serum 1:10, alumina plasma 1:10, RVV 0.004 mg. per milliliter phospholipid 0.1 mg. per milliliter, $CaCl_2$ 0.025 M. At the time indicated 0.1 ml. incubation mixture and 0.1 ml. $CaCl_2$ 0.025 M were added to the clotting substrate, with clotting times as noted.

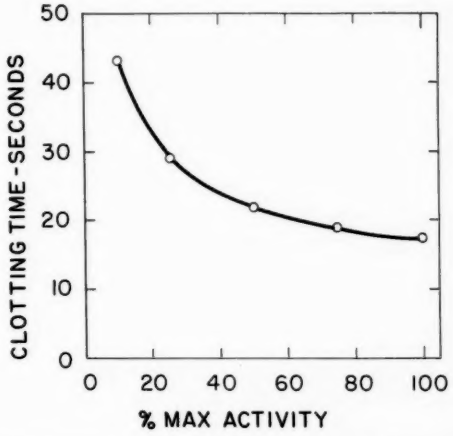

Fig. 1.—Per cent of maximum aliquot obtained during interval of maximum activity. The incubation mixture was composed of 0.2 ml. portions of each of the following: serum 1:10; alumina plasma 1:10; phospholipid 0.1 mg. per milliliter; RVV 0.004 mg. per milliliter; $CaCl_2$ 0.025 M. After 4 minutes, aliquots of 0.1, 0.075, 0.050, and 0.025 milliliter were removed and added to the clotting substrate. The clotting substrate had previously been diluted with the appropriate amount of buffer. The clotting times noted are plotted against the relative activity, assuming that the activity was proportional to the volume taken.

The conditions of Table I were adopted as standard. With different batches of reagents the curves differed slightly both as to the rate of development of thromboplastic activity and to the subsequent rate of loss. All the clotting times observed were converted to per cent activity based on the standard curve (Fig. 1).

Effects of Changes in the Concentrations of the Several Reagents.—The omission of any one of the reagents from the incubation mixture resulted in virtual absence of activity. Changes in the concentration of serum factor (either as serum or as DEAE-serum factor) caused distinct changes in the rate at which activity developed and in the amount of activity which ultimately arose (Fig. 2). Changes in the concentration of alumina plasma produced similar results (Fig. 3). Identical results were obtained with normal serum, purified serum fractions,

Factor IX (PTC)–deficient serum and VII-deficient serums. Stuart-deficient serum was inactive. Alumina plasma from all these deficient patients, as well as purified Factor V, behaved as does normal alumina plasma.

The effect of changes in the concentration of phospholipid is shown in Fig. 4. The initial rates are not affected, but the yield is clearly related to the concentration of phospholipid.

The system proved to be relatively insensitive to variation in the concentration of RVV (Fig. 5). A fourfold decrease in the RVV concentration produced a decrease in rate of only about 50 per cent and decreased the yield only a few per cent.

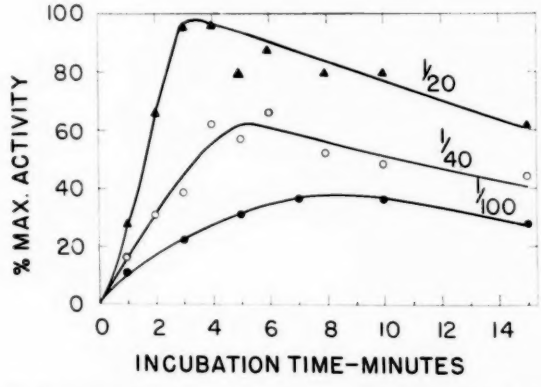

Fig. 2.—Effect of serum factor concentration on thromboplastic activity. Conditions for this experiment are the same described for Table I, except that the serum factor concentration (DEAE purified, twice serum concentration) was varied as indicated. The clotting times were converted to per cent activity by means of the standard curve.

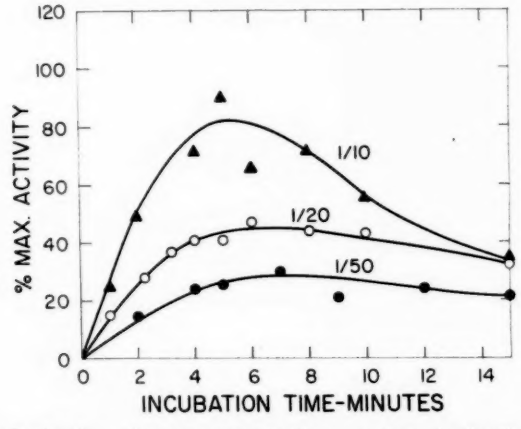

Fig. 3.—Effect of alumina plasma concentration on thromboplastic activity. Conditions for this experiment are the same as those given in Table I, except the concentration of alumina plasma was varied as indicated.

The effect of preincubating the various reagents with small amounts of RVV was investigated. In these experiments RVV at 0.0004 mg. per milliliter, 10 times lower than usual, was used. Each of the reagents was preincubated for 3 minutes with RVV, then the remaining reagents were added. All such combinations gave results identical to those found without preincubation. It was

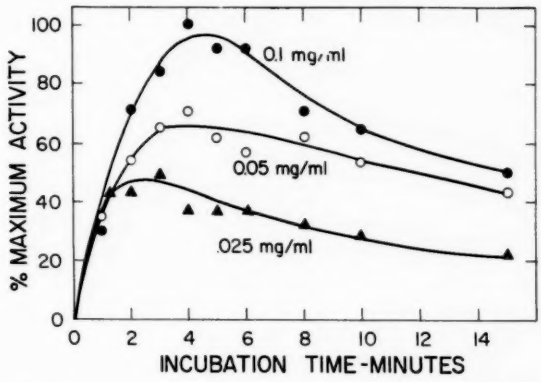

Fig. 4.—Effect of concentration of phospholipid on thromboplastic activity. Conditions for this experiment are the same as for Table I, except the concentration of phospholipid was varied as indicated on the figure.

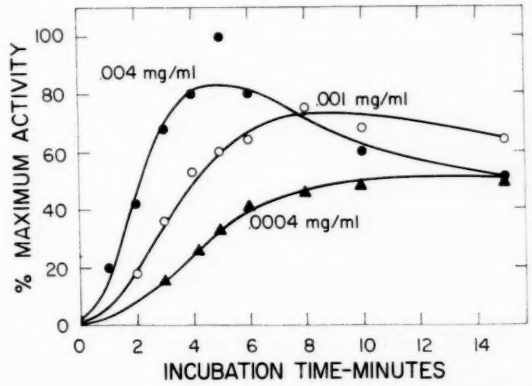

Fig. 5.—Effect of Russell's viper venom concentration on thromboplastic activity. Conditions for this experiment are as described in Table I, except that the RVV concentration was varied as indicated on the figure.

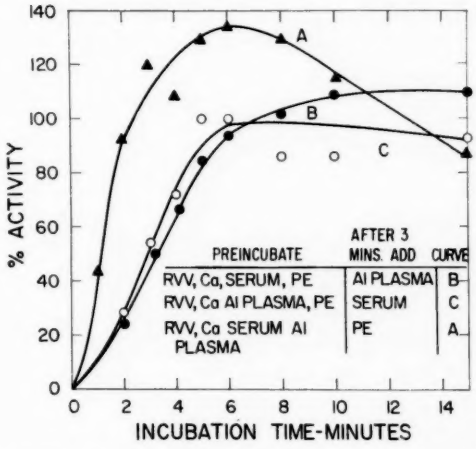

Fig. 6.—Effect of preincubation. Reaction mixture as described in Table I, with the exception that RVV was only 0.0004 mg. per milliliter. The standard curve was constructed with this lower concentration of RVV, and was similar to curves *B* and *C*. (PE indicates phosphatidyl ethanolamine.)

found that the only combination showing an effect of preincubation was that in which RVV, serum alumina plasma, and calcium were incubated, i.e., preincubation of all reagents except phospholipid. Typical results are shown in Fig. 6. The percentage activity was calculated from a standard curve obtained with RVV at 0.0004 mg. per milliliter, with no preincubation; other reagents were at concentrations indicated in Table I. It will be noted that preincubation gives a result comparable to that found for much higher concentrations of RVV without preincubation. Prolonging the preincubation to 20 minutes did not increase the maximum yield, but did increase the rate of formation by about 20 per cent.

DISCUSSION

In form, the reactions studied here closely resemble the thromboplastin generation reaction of Biggs and Macfarlane.[7] There are notable points of difference, however. Neither AHG nor PTC appear to be required for the RVV reaction, a fact noted earlier by Rapaport, Aas, and Owren.[9] The requirement for phospholipid is met by fraction 5 of Folch, but Biggs and Bidwell[10] have shown that this material is almost totally inadequate for the thromboplastin generation reaction. For these reasons the RVV reaction may proceed under conditions totally unsuited for the conventional thromboplastin generation reaction. The recognized factors known to be required are thus limited to Factor V[11, 12] and Stuart Factor.[13]

As in the thromboplastin generation test, a small clot usually forms in the incubation mixture at about the same time as the maximum thromboplastic activity is reached. The significance of this small amount of prothrombin present in the incubation mixture is not known, but the presence of considerably larger amounts of thrombin does not have any effect on the clotting times.[2] In a few exceptional instances with purified serum factor, and in the preincubation experiments without phospholipid, no clot was observed in the incubation mixture.

In an earlier paper in which we reported the reaction of Russell's viper venom, plasma was used as a substrate for measuring the thromboplastic activity.[2] Because of its large concentration of Factor V and Stuart Factor, plasma is not a suitable clotting substrate for the demonstration of the type of quantitative results which are reported here. Small amounts of these factors produce a large thromboplastic activity and the excessive quantities in the substrate completely obscure some of the changes which occur as individual reagents are varied. In the present work the Factor V content of the prothrombin was negligibly small. Although the Stuart Factor concentration was high enough in the undiluted prothrombin solutions to obscure some of the results, in the present experiments prothrombin was used in a sufficiently diluted form to overcome this interference, thus permitting the effect of changes in serum concentration to be measured.

A general feature of the data presented is that the production of prothrombin-converting activity is either accompanied by or immediately followed by destruction of the activity. It is likely that in some cases the relative rates of these two processes constitute the chief factor in determination of the maximum

activity, which we have referred to as the "yield." We mention this point, which has, of course, been dealt with more extensively by others, e.g., Biggs and Macfarlane,[7] because of the apparent dual role of Stuart Factor and Factor V in changing both the yield and the rate at which activity is generated.

Stuart Factor has been described by Fisch and Duckert,[14] as an enzyme affecting the rate of formation and by Hougie[15] as a substance which determines the yield of thromboplastic activity. In these two reports the experimental conditions were not comparable and it is conceivable that Stuart Factor may act in either way, depending on the circumstances. In this system it would appear that changes in Stuart Factor concentration change the rate and yield about equally.

We have been able to confirm the report of Hjort[11] that there is a very rapid activation of Factor V by RVV, but in spite of this fact the preincubation experiments reported here fail to disclose any effect of incubation of Factor V and RVV in the absence of serum factor. It is likely that some other reactions are rate limiting in the present experiments. O'Brien[12] also showed evidence for a reaction between RVV and Factor V. In his experiments the concentration of RVV was approximately 250 times that employed here, and again it appears likely that somewhat different reactions are rate limiting.

The preincubation experiments indicate that reduced quantities of RVV limit the rate of formation of prothrombin-converting activity rather than the final yield (Fig. 4) and that small quantities of RVV are as effective as larger amounts, provided more time is allowed (Fig. 6). This is good evidence for assumption of the catalytic role of some constituent in RVV, even though the identity of the specific substrate is unknown.

The nature of the reaction occurring in the preincubation mixture of RVV, Factor V, Stuart Factor, and calcium is obscure, but it affords additional evidence that some reaction can proceed in the absence of the phospholipid.[2] Although the reagents are entirely different, this product has a formal similarity to the Product I of Bergsagel and Hougie.[16] The formation of this hypothetical product is not, however, the only rate-determining step in the formation of thromboplastic activity. It may be seen from Fig. 6 that even if the formation of this product is allowed to proceed by preincubation there is still a relatively slow reaction between the product and the phospholipid. It is probable that in the other experiments reported here with higher concentrations of RVV, the principal time-consuming reactions are those with phospholipid.

The dependence of activity on the apparent interaction of Stuart Factor and Factor V (Figs. 2 and 3) makes it possible to assay each of these factors in a system such as this, provided a sufficient quantity of the other factor is present. In practice we have found that an assay system containing a 1 : 10 dilution of the factor not measured permits measurement of the other factor in dilutions corresponding to 1 : 50 of plasma. After a 3 minute incubation with RVV, phospholipid, and calcium, an aliquot is added to the clotting substrate. Standard curves may be prepared in the usual way, and unknown samples referred to some standard. Samples containing prothrombin are usually assayed at dilutions sufficient to reduce the prothrombin concentration to about 1 : 100 of plasma.

SUMMARY AND CONCLUSIONS

1. The formation of thromboplastic activity in an incubation mixture of adsorbed plasma, serum, phospholipid, calcium, and Russell's viper venom has been studied with respect to the effect of the concentrations of the various reagents used.

2. Variations in the concentration of the serum and plasma factors changed the rate at which thromboplastic activity developed and influenced the maximum activity reached.

3. Variation in the concentration of Russell's viper venom affected primarily the rate at which the activity developed, and was of less importance in determining the maximum activity. It appears that some constituent of Russell's viper venom acts catalytically in this reaction. Variations in the phospholipid concentration did not affect the rate at which thromboplastic activity developed, but did control the amount of activity which was attained.

4. Preincubation experiments indicate that there is first a reaction between Russell's viper venom and accessory factors in adsorbed plasma and serum; this product then reacts with the phospholipid in what is the main time-consuming reaction.

The accessory factors are identified as Factor V and Stuart-Prower Factor (Factor X). The system described permits an assay for these factors.

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DEOXYRIBONUCLEIC ACID SYNTHESIZING CELLS IN THE BLOOD OF MAN AND DOG EXPOSED TO TOTAL BODY RADIATION

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With the use of tritiated thymidine, which is incorporated only into new deoxyribonucleic acid, and the autoradiographic technique, it has been possible to show that cells capable of deoxyribonucleic acid synthesis and thus, presumably, of division are present normally in the blood. In the present investigations the number of these cells was followed serially in five men exposed to large doses of whole body radiation in an accident involving fissionable material at the Oak Ridge National Laboratory. Similar studies were performed on irradiated dogs to confirm and extend these observations. Following exposure the number of cells falls drastically within hours (normal values approximately 6 per cubic millimeter for human being; 60 per cubic millimeter for dog). The values then rise gradually, to attain levels exceeding normal approximately ten days after exposure of the human being. A second peak exceeding normal levels is noted at approximately forty days. The cells are all of the large mononuclear type.

The possible fate and function of these cells are discussed.

IT WAS shown previously, by means of tritiated thymidine and the autoradiographic technique, that cells capable of synthesizing deoxyribonucleic acid (DNA) are present normally in the peripheral blood.[1, 2] Thymidine is incorporated solely into newly formed DNA, and thus the presence of the labeled compound indicates ability of the cell to synthesize DNA and, presumably, to divide. The cells found were mononuclear. The number present in the normal human being is approximately 6 per cubic millimeter; the number is increased in some nonneoplastic[3, 4] and some neoplastic[3-6] diseases. In preliminary publications, it was shown that the number of these cells in the peripheral blood changes in both man[7] and dog[8] following whole body irradiation. In the present communication is presented the pattern of change in the number of circulating DNA synthesizing cells in human beings accidentally exposed to large doses of radiation from a critical assembly and in dogs exposed to different doses of x-radiation.

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MATERIALS AND METHODS

Human Exposures.—As a result of an accident involving nuclear fission which occurred in the Y-12 plant of the Oak Ridge National Laboratory in June, 1958,[7] five men were exposed to doses of total body radiation so high that hospital care was considered advisable. Details of the accident, and the clinical findings in the exposed individuals have been described previously.[7, 9, 10] The radiation consisted of a mixture of neutrons and gamma rays, and the total doses ranged from 236 to 365 rads, of which approximately one fourth was due to fast neutrons.

In connection with other hematologic studies, blood was drawn from each subject from Days 3 to 141 for the present studies.

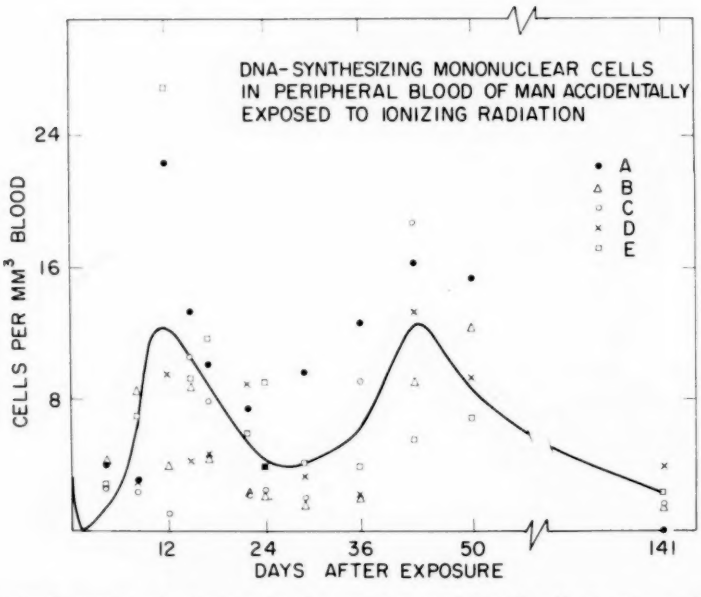

Fig. 1.—Sequence of changes in the number of labeled cells in the peripheral blood of human beings accidentally exposed to gamma and neutron radiations. All values on Day 3 were zero. The estimated total doses received, in rads, are as follows: *A*, 365; *B*, 270; *C*, 339; *D*, 327; *E*, 236.

Dog Exposures.—Four dogs were exposed to large doses of radiation as follows: Dogs 410 and 382 received bilateral radiation, 125 r from each side, totaling 250 r. Dog 368 received 350 r, unilaterally from the dorsal aspect. Dog 359 received 350 r, from the dorsal aspect, with the right hip and leg regions covered with ⅛ inch of lead (which reduces the dose below the shield to less than 3 per cent). Radiation factors were as follows: 250 KVP; 30 Ma.; filter 0.5 mm. copper, 1 mm. aluminum; TSD 110 cm.; machine output 21.2 per minute, measured at a position corresponding to the proximal skin surface of the animals. The doses given are those derived from the output dose rates at the proximal skin surface of the animal. The 250 r bilateral exposure is in the 30 day lethal range (one animal survived beyond 30 days; the other died on Day 17). The other two exposures would be considered less than, but approaching, the lethal range.

From all dogs, blood was drawn first hourly, then every few hours, and then at daily intervals for 16 days. From one dog (Dog 410, 250 r bilateral irradiation), occasional samples were taken from Days 17 to 30.

Labeling Technique.—The technique for in vitro labeling has been described previously.[1, 3] For both human beings and dogs, 4.5 ml. of blood was added to 0.5 ml. of stock solution containing tritiated thymidine, Na_2 EDTA, and dextran for acceleration of erythrocyte sedimentation. The final concentration of tritiated thymidine was 0.5 μc per milliliter, and the

specific activity of the material was 1.9 c per millimole. The blood was incubated for 1 hour at room temperature, at which time smears were made from cell concentrates and fixed in methyl alcohol. Films were developed after a 15 day exposure period, and were stained through the film with Giemsa stain. A total of 5,000 nucleated cells was enumerated at each of the points for both man and dog. At the time blood was withdrawn for the thymidine uptake studies, blood counts were performed in order to allow conversion of per cent labeled cells into absolute numbers of labeled cells per cubic millimeter.

RESULTS

Man.—The sequence of changes in the number of labeled cells in the peripheral blood of the human accident cases is shown in Fig. 1. In the normal human being[1-3] the labeled cells number approximately 6 per cubic millimeter. In an occasional individual, none are detected; in others, as many as 20 per cubic millimeter have been found. The cells are mononuclear, and of the types shown in Fig. 2 (cells in Fig. 2 are from the exposed patients). Some labeled cells resemble monocytes, others, immature lymphocytes, atypical lymphocytes, and plasma cells (none are typical plasma cells). On the third postexposure day, when studies were first done by this technique, the number of cells had fallen from the normal value of approximately 6 per cubic millimeter to zero. A rapid rise began on Day 4, with the number of cells in the peripheral blood being greater than normal from Days 8 to 18 (approximately). There was a second rise to above normal beginning approximately Day 28, and the number of cells in the peripheral blood was not significantly different from normal when the patients were last studied by this technique on Day 141, or 4½ months after the accident.

Fig. 2.—Cell types labeled with tritiated thymidine in the peripheral blood of human beings exposed accidentally to gamma and neutron radiations.

All of the labeled cells observed were mononuclear in type, and were approximately 10 to 15 microns in diameter. Most of the cells observed are of the types shown in Fig. 2. The nuclei of many showed a very dense chromatin pattern and the thin rim of cytoplasm was characteristically basophilic. In some cells, a slight indentation of the nucleus was observed; in a few cells the indentation was more pronounced, and a parinuclear "Hof" was evident in the region of the indentation. Essentially all cells seen were of the types described previously.[3]

Dog.—The sequence of changes in the number of labeled cells in the peripheral blood of the dog is shown in Fig. 3. Normally, approximately 50 labeled cells per cubic millimeter are present in the peripheral blood. These are of the types described for the normal human being; however, the predominant cells resemble immature or atypical lymphocytes. Within minutes of exposure,

the number of labeled cells increased to about double the normal value, followed by a very rapid decrease to virtually zero by the end of 24 hours. The number increased over a period of the next several days, then decreased, and perhaps began a secondary rise on approximately Day 16. In one dog given bilateral irradiation and observed beyond Day 16, a secondary rise occurred around Day 22, with a subsequent fall (data not shown in Fig. 3). The absolute numbers in the peripheral blood from approximately the second to the twelfth day were considerably lower in the animals that received bilateral irradiation than in the animals that received either whole body irradiation unilaterally, or unilateral irradiation with one hind limb shielded. However, the general shape of the curve was the same in all four animals.

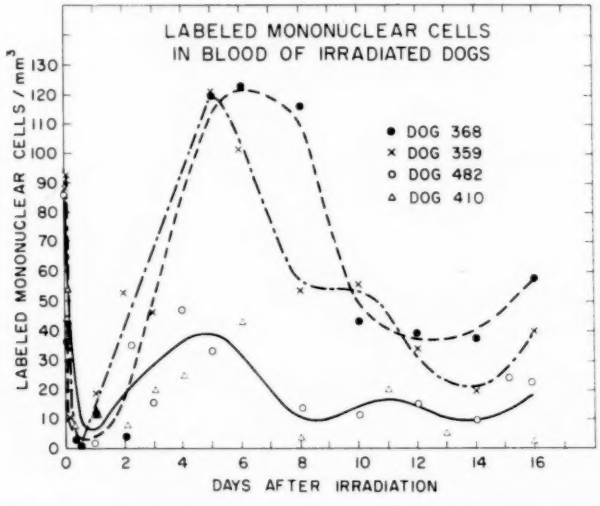

Fig. 3.—The sequence of change in the number of labeled cells in the peripheral blood of dogs exposed to 250 KVP x-radiation. Dogs 410 and 382 received 250 r bilaterally; Dog 368 received 350 r from the dorsal aspect; Dog 359 received 350 r from the dorsal aspect, with right hip and leg regions shielded.

The morphology of the labeled cells observed in the irradiated dogs was no different from that normally observed.

DISCUSSION

Although it is apparent from the data that considerable variation occurred from individual to individual, the changes in each subject were of a regular nature, and the average curves for both dog and man were similar. In both man and dog, the number of labeled cells had fallen drastically within at most a few days of exposure, and the dog data indicated that the fall occurs within the first 24 hours of exposure. In man, there were two subsequent elevations in the number of cells in the peripheral blood, which occurred on approximately the twelfth and fortieth days. A secondary rise occurred in one dog given bilateral irradiation and studied beyond 16 days. After apparently complete recovery the number of cells had returned to essentially normal values. These data indicate a definite pattern of change in the number of labeled cells following heavy exposure of both man and dog.

The results are given in terms of the absolute number of labeled cells per cubic millimeter, independent of the precise cell type. Because of the small number of labeled cells involved, further breakdown into subcategories was not warranted. It would be valuable to know if only the number of labeled cells changed, or if the total number of morphologically similar cells, labeled or unlabeled, changed as well. Although accurate categorization is difficult in the stained filmed preparations, this was attempted in one animal (Dog 410). The numbers of morphologically similar, labeled and unlabeled cells changed in a parallel fashion, indicating that the differences following irradiation relate to all cells of that category and not only to those in DNA synthesis.

It has been shown that tritiated thymidine is incorporated in vivo and in vitro solely into DNA of cells that are in DNA synthesis and hence have the potential for further cell division.[11] It is thus assumed that the mononuclear blood cells found to be capable of incorporating tritiated thymidine have a proliferative potential, but that they do not necessarily divide within the blood stream. The origin, fate, and function of these cells and the reason for the pattern of change following irradiation are not clear.

It is not definitely established whether alterations in the number of labeled cells in the peripheral blood after exposure parallel changes in hemopoiesis or lymphopoiesis. In general, in man, for the dose ranges considered, the blood neutrophils may rise transiently within hours of exposure, and then fall gradually. The low point is reached in the third week or later, depending on dose, and recovery of survivors begins in approximately the fifth week. The lymphocyte count falls to the lowest values attained (20 to 40 per cent of normal) within 24 hours, and remains at this low value for some weeks before recovery begins. However, the curves of serial lymphocyte counts in both the Oak Ridge and Yugoslavian accident cases[7, 10, 12] indicate questionable rises around Days 15 and 40. The data on patients exposed in two Los Alamos accidents reveal no such changes.[13] From data on animals[14] and on the Oak Ridge patients,[7, 10] it is concluded that an "abortive rise" in peripheral blood leukocytes may occur some days after exposure. It is known[15] that the mitotic index of the bone marrow also rises after an initial fall, and that many of the mitotic figures are obviously abnormal at the time of this abortive rise. Thus the changes observed in the number of labeled blood cells resemble to some degree those seen in lymphopoiesis and medullary hematopoiesis, and it is not clear whether they are related to changes in either of these hematopoietic systems. It is of course possible that they are related to neither, and that the cells belong to a separate system with a different site of formation.

The curve following exposure of a dog with one limb shielded is similar in shape to that of the animal receiving the same dose of radiation, but without shielding of the limb. The same type of curve has been noted in patients receiving partial body exposure in the course of clinical radiotherapy.[6] The absolute values in the dog which received bilateral radiation were considerably less than those in either dog which received unilateral radiation. It should be noted that bilateral radiation is more effective than is unilateral radiation, and the

"biologically effective" dose, in terms of potential mortality, was higher with the dogs receiving bilateral radiation than with either dog receiving unilateral radiation.

It is tempting to speculate that the changes in the numbers of labeled cells following irradiation may be related to marrow degeneration and regeneration, and that the cells may have a multipotential hematopoietic function. Some support for this thesis can be derived from the well-known fact that shielding of hematopoietic tissues under various conditions in the irradiated animal results in accelerated regeneration of the bone marrow and a decrease in mortality. This could result from possible transfer of a humoral substance or of cells capable of proliferation, neither of which possibility has been established. Support for the cell transfer hypothesis is seen in the fact that radiation protection has been obtained in mice by means of blood transfusions from animals with a leukemoid reaction.[16-18] Efforts in this laboratory to demonstrate proliferation of blood cells labeled with tritiated thymidine and transfused into normal or irradiated recipient animals have not been successful to date[2]; however, the data are not considered to be definitive evidence against such proliferation under some circumstances.

Labeled cells of the types described in the peripheral blood are observed in large numbers in pleural and peritoneal effusions of some cases[6] with which a direct association with hemopoiesis would not be expected. Some of the labeled cells have been demonstrated in this laboratory to be phagocytic. Thus it is possible that at least some of the labeled cells found in the blood may be concerned with inflammation and defense mechanisms, and their apparent proliferative potential may be related to an ability to provide rapidly a mechanism for building up local defenses.

SUMMARY

1. Mononuclear cells capable of deoxyribonucleic acid synthesis, as determined by use of tritiated thymidine and autoradiography, were found in the peripheral blood of five patients accidentally exposed to ionizing radiation, and in four x-irradiated dogs.

2. The absolute number of these labeled cells undergoes apparently characteristic changes in the postirradiation period, with two peaks above normal in the human being around Days 8 to 10 and 35 to 40.

3. Some grounds for relating the changes noted to changes in the lymphopoietic or the medullary hematopoietic system can be seen; however, the fate and function of these cells is not clear at present.

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EFFECT OF MERCAPTANES IN COLD AGGLUTININ DISEASE

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In cold agglutinin disease the untoward effects can be attributed chiefly to high serum concentration of a macroglobulin with S_{20} value of 15 to 19. Treatment, including high environmental temperature, ACTH, and HN_2 has not been rewarding. Another therapeutic approach has been explored. Other investigators demonstrated that macroglobulins are depolymerized by mercaptanes. Therefore, the effect of mercaptanes upon the macromolecular cold agglutinin in idiopathic cold agglutinin disease was examined. The subject of this study was a 65-year-old white man suffering from intermittent severe hemolytic anemia which required frequent transfusions. Serum from this patient contained a cold agglutinin characterized by S_{20} value of 15.5; electrophoretic mobility of γ_1-β_2-globulin; intense periodic acid–Schiff staining; negative Sia and formolgel tests; cold agglutinin titer of 1:500,000 to 1:1,000,000 at 5° C. with thermal amplitude extending to 30° or 32° C. Serum was incubated with cysteamine, vitamin B_6-SH, penicillamine, and penicillin G for 30 minutes. The titer was substantially lowered in all samples except the control. Penicillamine (500 mg. t.i.d.) was administered orally for 10 days. There followed a marked decrease in the titer of cold agglutinins and acid cold hemolysins, with reduction of thermal amplitude. At cessation of treatment, the titers again rose. Since lowering of cold agglutinin titer corresponds with reduction of thermal amplitude (e.g., 20° C.) and cold sensitivity, further exploration of this biochemical approach is warranted.

AMONG the disorders associated with cold intolerance, cold agglutinin disease[1] is one of the more challenging. Although this condition has been recognized since 1873,[2] it has been frequently mistaken for other cryopathies,[3,4, 36] such as (syphilitic) paroxysmal cold hemoglobinuria, Raynaud's disease, or cryoglobulinemias.

No effective treatment for this disorder, other than advising the patient to remain in warm surroundings, has been reported.

Since cold agglutinins have been demonstrated to be macroglobulins[8, 9, 14] which are sensitive to sulfhydryl moieties,[18-20] the effect of mercaptanes upon their activity in vitro and in vivo was explored.

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MATERIALS AND METHODS

The experimental material was provided by a 65-year-old white man with a 6-year history of cold agglutinin disease. There was a marked increase in reticulocytes, fecal urobilinogen excretion, and shortening of the Cr^{51} survival time of the erythrocytes, which necessitated frequent transfusions of blood preheated to 40° C. The studies were performed while the patient was in the hospital. In order to establish base-line conditions the patient was exposed to a temperature of 20° C. for 30 minutes daily. The Donath-Landsteiner test[12] was negative. The modified Ehrlich's test[13] revealed hemolysis at 20° C., but not at 40° C. Smears prepared during the test revealed erythrophagocytosis at 20° C. The Venereal Disease Research Laboratory test was negative. The serum was examined electrophoretically and ultracentrifugally.

The influence of mercaptanes upon the serum agglutinins was examined in vitro according to the following methods:

1. To 2.0 ml. of *fresh* cold agglutinin serum, 0.2 ml. of 0.9 per cent sodium chloride was added. This control sample was incubated at 37° C. for 30 minutes, and the cold agglutinin titers were determined at 5° C., according to Dacie's method.[12] Gradation from 1 to 4+ was employed: 1+ indicating slight macroagglutination, 2+, moderate, 3+, strong, and 4+, very strong agglutination, the entire erythrocyte mass being one firm agglutinate. To a second serum sample 20 mg. of d,l-penicillamine (d,l-dimethylcysteine*) dissolved in 0.2 ml. of 0.9 per cent sodium chloride solution was added. A third sample was treated with 20,000 units of penicillin G potassium (Squibb) dissolved in 0.2 ml. of 0.9 per cent sodium chloride. To a fourth aliquot of the serum, 0.2 ml. of one per cent solution of cysteamine (beta-mercaptoethylamine†) in 0.9 per cent sodium chloride was added.

2. These experiments were repeated with serum stored at −20° C. for 4 months. In addition to these aforementioned agents, 20 mg. of vitamin B_6-SH (mercaptopyridoxine, 3-mercaptomethyl-4-oxymethyl-5-oxy-6-methylpyridine‡) in 0.2 ml. of 0.9 per cent sodium chloride was added to a fifth serum sample. Incubation period was 2 hours at 37° C.

3. Serum treated with penicillamine in the same fashion as described in (1) and (2) above was examined by paper electrophoresis (Spinco Durrum, Model R, Series C) immediately and 24 hours after incubation.

4. Penicillamine-treated serum (same method as described above, but with 20 minutes of incubation) was examined in the ultracentrifuge.§ [The treated serum was diluted 1:10 in buffered 0.9 per cent sodium chloride solution, pH 7.0, at 20° C., 56,100 r.p.m. (Spinco Model E)]. The penicillamine-treated serum was examined immediately after incubation, whereas the control sample was stored at 20° C. for 60 minutes before ultracentrifugation.

The effects of d,l-penicillamine and vitamin B_6-SH were examined in vivo:

1. D,l-penicillamine was given in a dosage of 500 mg. three times a day orally for 10 days and the cold agglutinin titers were determined twice weekly (Dacie's method).[12] The titers were recorded in a 1 to 4+ gradation. In addition, the mean titers (M_t) were calculated and graphically presented as follows:

$$\left(M_t = \frac{\text{titer (1+)} + \text{titer (2+)} + \text{titer (3+)} + \text{titer (4+)}}{4}\right).$$

All titers were determined in duplicate.

2. The effect of intravenously administered vitamin B_6-SH was explored. Mercaptopyridoxine was given in a dosage of 0.3 Gm. twice a day. The titers in this experiment were determined according to Schubothe.[15]

*Aldrich Chemical Company, Inc., Milwaukee, Wis.

†K & K Laboratories, Inc., Jamaica, N. Y.

‡E. Merck A.G., Darmstadt, Germany.

§Ultracentrifugal examinations were performed by Dr. Alexander H. Woods, University of Oklahoma Department of Medicine, Oklahoma City, Okla., and Dr. G. Lospalluto, University of Texas, Southwestern Medical School, Department of Biochemistry, Dallas, Texas.

The influence of the mercaptanes upon simultaneously occurring acid-cold hemolysins was studied. The determination of acid-cold hemolysins was performed at 22° C. and pH 6.5, according to Schubothe's method.[15]

Since high titers of cold agglutinins produce an acquired extracorpuscular hemolytic anemia, the effect of in vivo administration of d,l-penicillamine and vitamin B_6-SH upon the Cr^{51} survival time of erythrocytes was examined. Labeling of erythrocytes with Cr^{51} was performed in the usual manner.[21]

RESULTS

1. Electrophoresis of the serum (electroserogram, ESG) revealed the presence of a homogeneous fraction at the γ_1-β_2 range, representing the cold agglutinins (Fig. 1). This fraction was strongly P.A.S. positive.

In the ultracentrifuge, the cold agglutinins were found to be macroglobulins, characterized by 15.5 S_{20} (uncorrected for protein concentration) (Fig. 2).

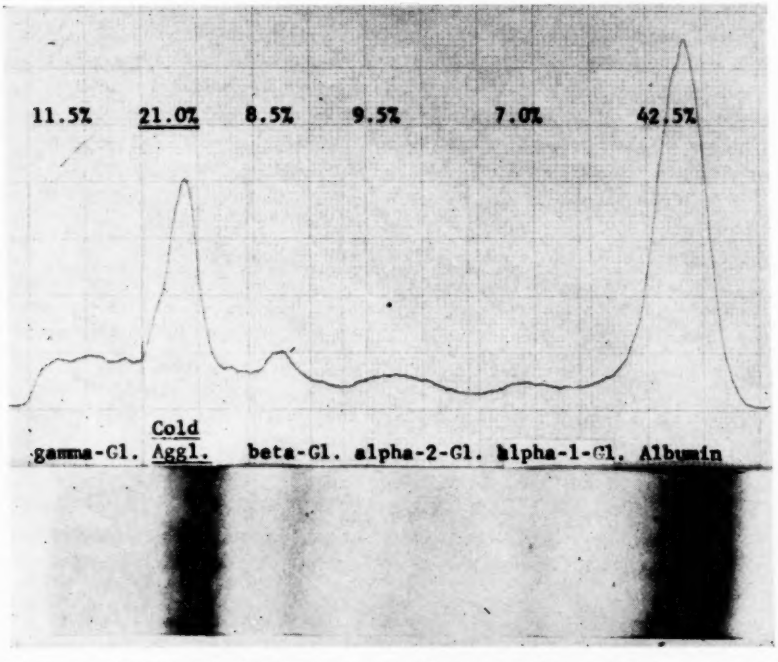


Fig. 1.—Serum electrophoresis (electroserogram, ESG) of cold agglutinin serum and P.A.S. staining.

2. a. The addition of penicillin G potassium, d,l-penicillamine, and cysteamine to fresh serum resulted in the following changes of the cold agglutinin titers at 5° C.: Penicillin G decreased the titers from 1:1,048,576 to 1:262,144 (1+); from 1:131,072 to 1:8,192 (2+); from 1:16,384 to 1:4,096 (3+); the titer of 1:1,024 (4+) remained unchanged. D,l-penicillamine reduced the titers from 1:1,048,576 to 1:32,768 (1+); from 1:131,072 to 1:4,096 (2+); from 1:16,384 to 1:1,024 (3+); and from 1:1,024 to 1:32 (4+). The corresponding values for cysteamine were: unchanged 1+ titers (1:1,048,576); reduction from 1:131,072 to 1:2,048 (2+); from 1:16,384 to 1:1,024 (3+); and from 1:1,024 to 1:32 (4+).

b. Incubation of aged serum with penicillin G potassium, d,l-penicillamine, cysteamine, and vitamin B_6-SH resulted in the following changes of the

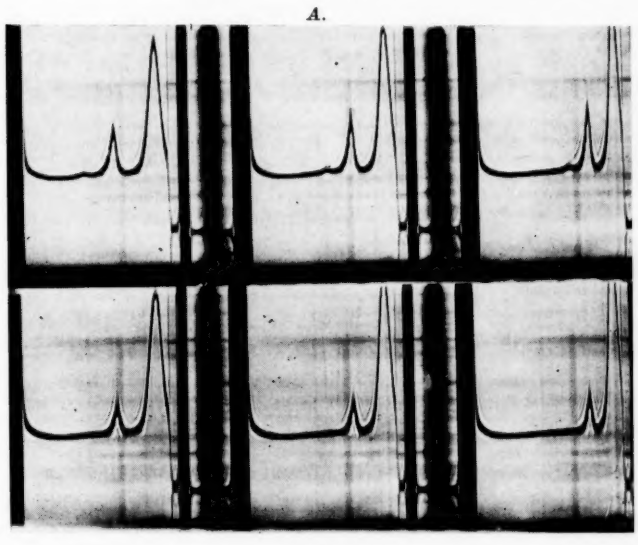


Fig. 2.—Ultracentrifugal pattern of cold agglutinin serum. The fractions were photographed at 4-minute intervals (from right to left) (12, 16, and 20 minutes). *A*, Control sample. *B*, Penicillamine-treated serum.

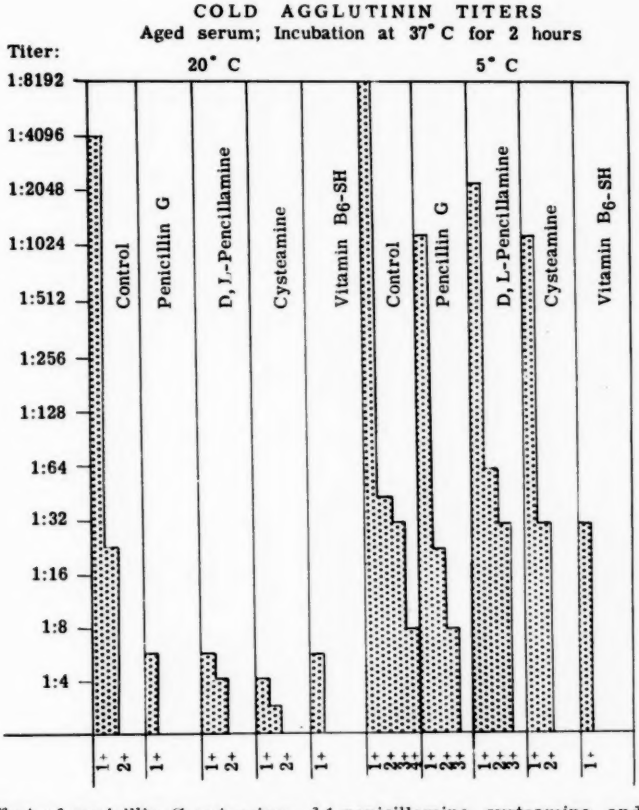


Fig. 3.—The effect of penicillin G potassium, d,l-penicillamine, cysteamine, and vitamin B_6-SH upon the cold agglutinin titers of aged cold agglutinin serum (20° and 5° C.).

cold agglutinin titers: Penicillin G decreased the cold agglutinin titers (at 5° C.) from 1:8,192 to 1:1,024 (1+); from 1:64 to 1:32 (mean values) and to 1:32 to 1:16 (mean values) (2+); from 1:32 to 1:8 (3+); and from 1:8 to 0 (4+).

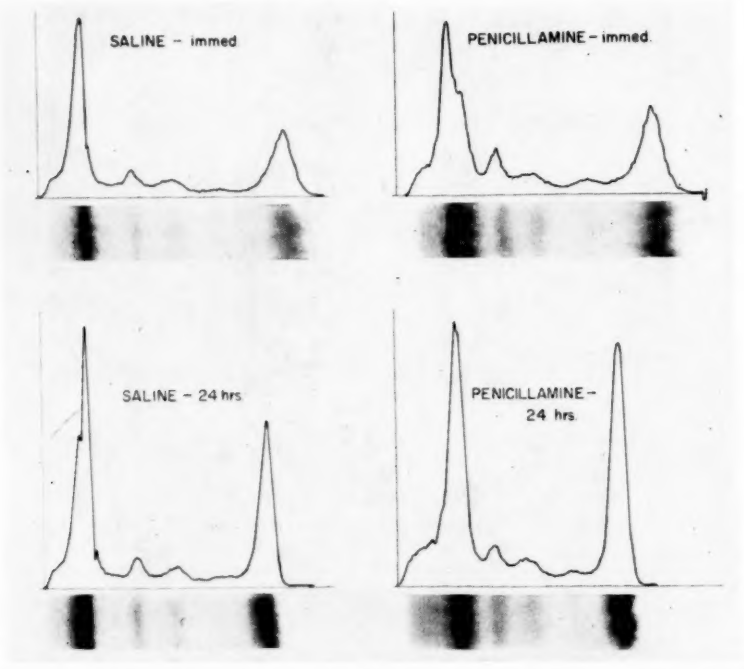


Fig. 4.—Electroserogram of control serum and penicillamine-treated serum samples. (See text.)

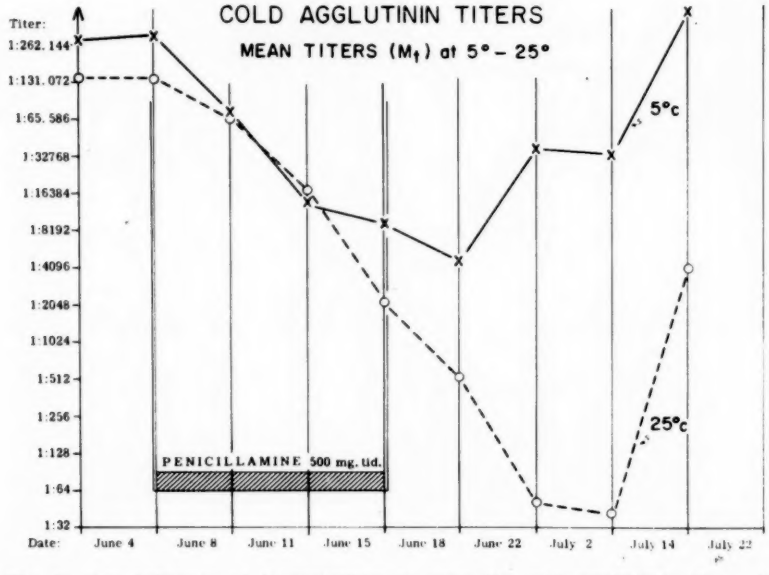
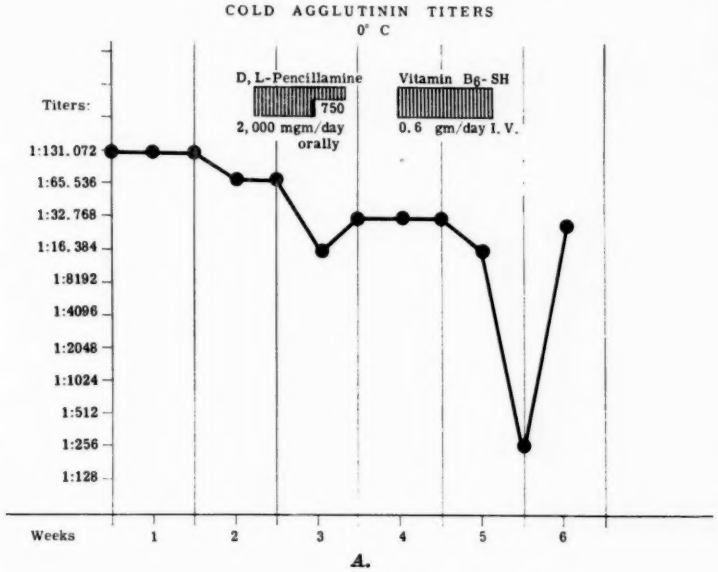


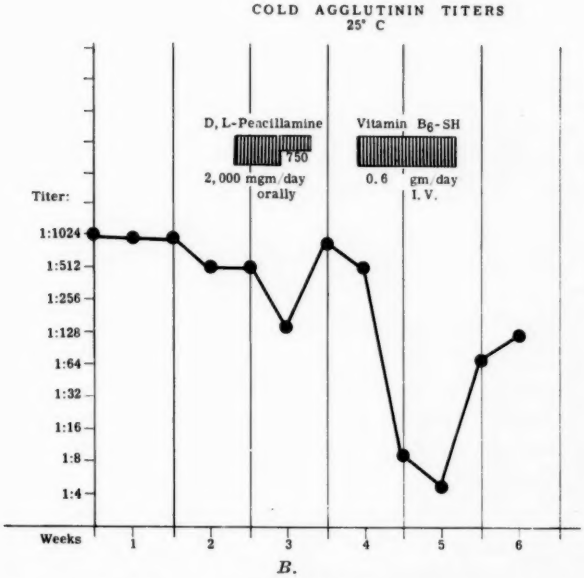
Fig. 5.—The in vivo influence of d,l-penicillamine upon the cold agglutinin titer. (For mean titers, see text.)

The corresponding values for d,l-penicillamine, cysteamine, and mercaptopyridoxine are as follows: 1:2,048, 1:1,024, and 1:32 (1+); 1:64, 1:32, and 0 (2+); 1:32, 0, and 0 (3+); and 1:32, 0, and 0 (4+) (Fig. 3).

c. Penicillamine-treated serum revealed a splitting of the gamma globulin peak (Fig. 4). The previously homogeneous γ_1-β_2 peak was converted into a heterogeneous gamma globulin fraction. Twenty-four hours later (after storage at 25° C.) again a homogeneous γ_1-β_2 globulin peak was found. The control sample did not reveal any changes in the globulin range.



A.



B.

Fig. 6*A* and 6*B*.—The in vivo effect of vitamin B_6-SH upon the cold agglutinin titer at 0° and 25° C. (Effect of short-term treatment with d,l-penicillamine recorded on left.)

d. The penicillamine-treated sample revealed a significantly lowered macromolecular fraction (Fig. 2, *B*) as compared to the control sample (Fig. 2,*A*). The 15.5 S_{20} (uncorrected for concentration) component (cold agglutinins) is reduced from 17.93 per cent of total serum proteins to 9.97 per cent, e.g., 44.4 per cent. The 23 S_{20} component present in the control sample (1.84 per cent of total serum protein) is absent in the penicillamine-treated sample. The total reduction of the two macromolecular fractions is thus 49.6 per cent.

3. a. The effect of d,l-penicillamine in vivo: The cold agglutinin titers decreased in succession after 10 days of treatment from 1:262,000 at 5° C. to 1:4,096, and from 1:131,000 at 25° C. to 1:32 36 days after initiation of treatment. On the forty-fourth day, the titers had returned toward pretreatment levels (Fig. 5).

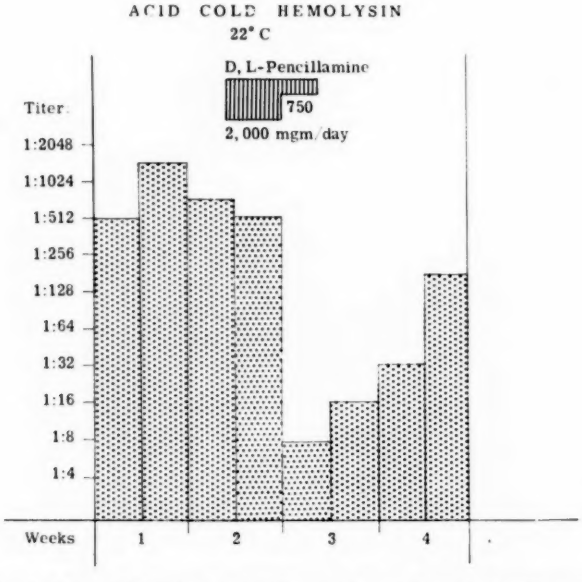


Fig. 7.—The effect of d,l-penicillamine upon the acid-cold hemolysin titer following oral administration.

b. Intravenous injection of vitamin B_6-SH was also followed by a marked decrease of the cold agglutinin titer, namely, from 1:32,000 at 0° C. to 1:256 (Fig. 6*A*), and from 1:1,024 at 25° C. to 1:4 (Fig. 6*B*).

The reduction of the cold agglutinin titers following penicillamine and mercaptopyridoxine treatment was associated with a shortening of the thermal amplitude. Whereas, before treatment, agglutination occurred at 32° C. (titer 1:2), immediately after treatment there was no agglutination observed between 25° and 32° C.

4. The titers of the acid cold hemolysins were reduced from 1:512 and 1:1,024, respectively, to 1:8 (Fig. 7).

5. Influence of mercaptanes upon the Cr^{51} survival time of erythrocytes: During the periods of treatment of d,l-penicillamine and vitamin B_6-SH, a

change in the curves was observed which suggested a lengthening of the survival time of the erythrocytes (Fig. 8). After cessation of the administration of the mercaptanes, the pretreatment half-life of approximately 11 days reappeared. (Normal half-life in our laboratory: 29 to 30 days.) There was an associated decrease of reticulocytes. During and immediately following the treatment periods, the patient's condition improved subjectively; the fatigue was abolished, the appetite increased, and the pallor receded.

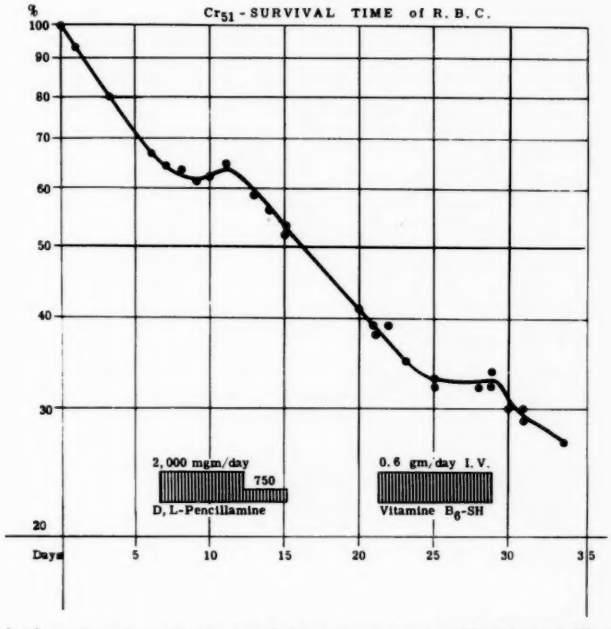


Fig. 8.—Cr^{51} survival curve of erythrocytes during treatment with d,l-penicillamine and vitamin B_6-SH.

DISCUSSION

Under physiologic conditions human serum contains globulins which agglutinate autologous, homologous, and certain heterologous erythrocytes at low temperature ranges. The normal serum titers of cold agglutinins at 0° C. vary between 1:4 and 1:128.[3, 35] Cold agglutinins are macroglobulins[8, 9, 14] with a molecular weight of approximately one million and 15.2-19 S_{20}.[22, 32] They are composed of globulin subunits, linked by disulfide bridges, and should therefore be sensitive to reagents known to reduce such disulfide bridges as sulfhydryl compounds or mercaptanes. Electrophoretically, cold agglutinins are found in the γ_1-β_2 range.[8, 9, 32]

Certain virus diseases are associated with a transient increase of cold agglutinins.[4, 5, 35] In some lymphomas and related disorders the cold agglutinin titers may rise as high as 1:64,000.[7] The highest increase of cold agglutinins is observed in elderly persons suffering from the so-called cold agglutinin disease,[7] with the clinical triad of acrocyanosis, hemolytic anemia, and hemoglobinuria. Here, the titers may be as high as 1:1 million at 0° C. Higher titers should cause suspicion of faulty titration technique. In order to prevent carry-over,

clean pipettes must be used for each titration step; furthermore, the determination of the titers should be carried out under circumstances that permit strict maintenance of the incubation temperature.

The three conditions associated with increases of cold agglutinins (acute increase in certain virus infections; subacute increase in some lymphomas; and chronic increase in idiopathic, cold agglutinin disease) constitute the *cold agglutinin syndrome.*[36] The relationship of this syndrome to the cold hemolysin syndrome and the other cryopathies is discussed elsewhere[36] (Table I).

TABLE I. COLD AGGLUTININ SYNDROME

		COLD AGGLUTININ SYNDROME		
STATE	PHYSIOLOGIC	CERTAIN VIRUS INFECTIONS (E.G., PRIMARY ATYPICAL PNEUMONIA)	MALIGNANT LYMPHOMAS	IDIOPATHIC COLD AGGLUTININ DISEASE
Cold agglutinin titers	1:4 to 1:128	1:256 to 1:64,000	1:256 to 1:128,000	1:8,000 to 1:1 million
Duration of increased titers		Acute, transient	Subacute (duration of underlying disease)	Chronic

The relationship between the cold agglutinin titer and the effective temperature range is depicted in the temperature-titer-curve of Fig. 9. Two points are demonstrated:

The normal titer is increased moderately in the presence of certain virus diseases, and markedly in the cold agglutinin disease (ordinate).

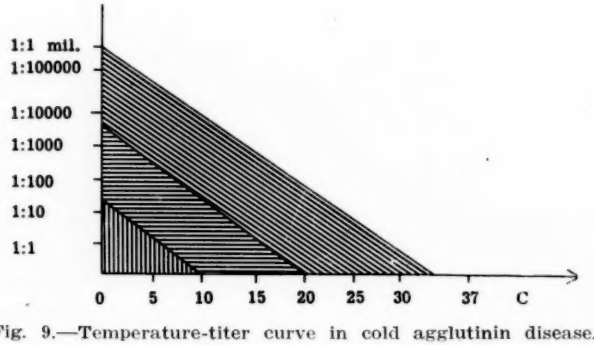


Fig. 9.—Temperature-titer curve in cold agglutinin disease.

The temperature range in which cold agglutinins are active is a function of the titer: the higher the titer, the higher the thermal range or amplitude (abscissa). Under physiologic conditions, agglutination of red blood cells will occur only below 10° C., in primary atypical virus pneumonia it may occur at temperatures up to approximately 20° C.; whereas, in cold agglutinin disease, hemagglutination (and hemolysis) may occur up to 30° or 32° C., which is the normal skin temperature range.

In cold agglutinin disease two immunologic phenomena are encountered: (1) the nonspecific nature of *agglutinating* properties (cold agglutinins) with respect to blood groups or species of erythrocytes and (2) the *hemolytic* effects

upon human erythrocytes. The latter occurs optimally at 22° C. and at pH 6.5 to 6.8[12] (acid-cold hemolysins). In the majority of patients with cold agglutinin disease there is a relationship between the titers of cold agglutinins and acid-cold hemolysins in that they parallel each other to a certain degree. Electroserogram and other examinations almost always reveal the presence of only one protein fraction which contains the antibodies. For these and other reasons[7, 17] it may be assumed that the macromolecular globulin found in the γ_1-β_2 position in the electroserogram is responsible for both the agglutinating and hemolysing effects in the cold agglutinin syndrome.[12, 15]

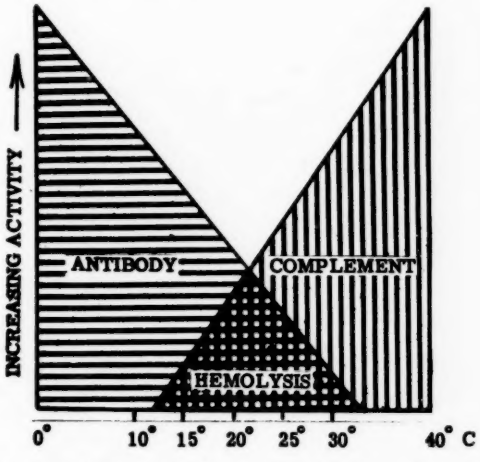


Fig. 10.—Temperature hemolysis curve (after Schubothe). (For description, see text.)

The agglutinating effect is independent of the presence of serum complement (*complete antibodies*) and the maximal agglutinating activity is observed at 0° C., with this effect decreasing as the temperature rises (*monothermic, monophasic cold agglutinins*) (Fig. 10). The hemolytic activity of the cold agglutinins is dependent upon the presence of serum complement (*incomplete, acid-cold hemolysin*).[37] In contrast to the hemolysins found in chronic paroxysmal (syphilitic) hemoglobinuria, the acid-cold hemolysin of cold agglutinin disease is monophasic (*monothermic*). Whereas in cold agglutinin syndrome the binding of the amboceptor and the hemolytic effect can take place at one temperature range, e.g., 20° C., the abnormal complement-dependent hemolysin of the cold hemolysin syndrome (paroxysmal cold hemoglobulinuria: syphilitic, or non-syphilitic) usually requires cooling to below 20° C. to permit absorption by the erythrocytes and subsequent warming to above 25° C. for hemolysis to occur (*biphasic, bithermic cold-warm hemolysin*). Both the agglutinating effect and the binding of the hemolytic amboceptor onto the erythrocytes are within certain limits reversible in both conditions. The differential diagnosis can be made (in vitro) by the Donath-Landsteiner test[12] and (in vivo) by the Ehrlich finger test as modified by Schubothe.[13]

The thermal range for optimal activity of the acid cold hemolysins is depicted in Fig. 10, modified from Schubothe,[7] demonstrating the temperature-hemolysis curve: The shaded area on the left represents the zone of acid-cold

hemolysin amboceptor binding, the shaded area on the right represents the zone of temperature dependent activity of complement. The shaded triangular area in the middle demonstrates the zone in which the complement is active and in which the binding of the amboceptor takes place. This zone is identical with the temperature range in which hemolysis occurs, with maximum effect at 22° C. The acid-cold hemolysin titers found in cold agglutinin disease usually range between 1:320 and 1:2,048.

Deutsch and associates,[18] and Glenchur and co-workers[19] have demonstrated that mercaptanes can depolymerize macroglobulins. Heimer and associates[10] have demonstrated that the macroglobulins representing the rheumatoid arthritis factors (19 and 22 S components) can be depolymerized to a 7 S unit with cysteine. This reduction was associated with loss of the hemagglutination properties. Subsequent reaggregation of the depolymerized fractions did not restore immunologic activity. Deutsch and Chan[16] were also able to degrade the complete (macromolecular, saline type) Rh antibodies and the A and B isohemagglutinins by means of mercaptoethanol. Here also the depolymerization was associated with marked diminution or complete loss of antibody titers. Reaggregation of the monomer units to a macromolecular component did not restore the Rh activity. Isliker[20] treated physiologic 19 S alpha-2-macroglobulins with cysteamine; the ultracentrifugal pattern revealed a 6 S component. In contrast to the effect upon macroglobulins, similar treatment of normal 7 S gammaglobulins produced no change of the ultracentrifugal or viscosity patterns. The in vitro and in vivo treatment of a macrocryogelglobulin with d,l-penicillamine and mercaptopyridoxine, in our laboratory,[23, 31] has also resulted in a marked decrease of the relative viscosity.

Repolymerization of degraded macroglobulins may result in increased viscosity often exceeding the original values.[20, 23, 31]

It has also been demonstrated that properdin can be converted from a 23-25 S component to a 6 S fraction by cysteamine.[20] Recently, Fudenberg and Kunkel[22] reported that mercaptanes produced depolymerization of the macromolecular cold agglutinins (in vitro) from a case of severe cold agglutinin disease. The resulting monomers were immunologically inactive.

These biochemical studies permit characterization of 4 essential features of macroglobulins: (1) Macroglobulins can be depolymerized by a variety of mercaptanes; (2) specific immunologic properties of macromolecular antibodies are abolished by depolymerization; (3) despite reaggregation of the monomers to macroglobulin components there is no restoration of the intrinsic immunologic activity; (4) nonspecific, nonimmunologic, physical properties of macroglobulins (e.g., viscosity) do reappear after reaggregation. There is no effective treatment for the cold agglutinin disease except to advise the patient to avoid cold surroundings. Splenectomy,[6, 33] ACTH,[7] steroids,[7, 33] and nitrogen mustard[7] have not been beneficial. The abolition of specific immunologic characteristics of macromolecular antibodies by depolymerization suggested investigation of a biochemical approach to cold agglutinin disease.

The treatment of fresh cold agglutinin serum with penicillin G potassium, d,l-penicillamine, and cysteamine produced a marked decrease of cold agglutinin titer with penicillamine, a moderate reduction with penicillin, but no effect with cysteamine. Aged serum, however, responded uniformly with a sharp decrease in titers following the use of all three compounds and mercaptopyridoxine. Penicillamine produced widening and notching of the previously homogeneous β_2-γ_1 cold agglutinin fraction, as was seen when the fraction was examined electrophoretically immediately after incubation. Upon standing for 24 hours at room temperature, the homogeneity of the cold agglutinin fraction was restored. In the ultracentrifuge, penicillamine treatment of cold agglutinin was shown to result in partial depolymerization. These reproducible reductions of the cold agglutinin fractions and titers were also observed to occur in vivo after administration of penicillamine and mercaptopyridoxine. The administration of d,l-penicillamine was followed by successive reduction of the cold agglutinin titers from 1:262,000 to 1:4,096 at 5° C. and from 1:131,000 to 1:32 at 25° C. (Fig. 5). On the forty-fourth day, the titers had returned toward pretreatment levels. Intravenous injection of mercaptopyridoxine also produced a marked decrease in cold agglutinin titer (Figs. 6, *A* and *B*). The cold agglutinin titers began to rise 3 days after cessation of treatment and reached pretreatment values in approximately 2 weeks.

Simultaneously with the cold agglutinin titers, the acid-cold hemolysin titers decreased sharply: from 1:512 and 1:1,024, respectively, to 1:8. Following treatment, the titer rose steadily to pretreatment levels within 2 weeks.

The effect of mercaptanes on Cr^{51} survival times of erythrocytes was determined concomitantly. Both penicillamine and mercaptopyridoxine altered the survival curve in a direction which suggested lengthening of survival time of the erythrocytes (Fig. 8). Both treatment periods were brief and were followed by prompt resumption of the pretreatment survival curve, which indicated a half-life of approximately 11 days.

The relatively long recovery phase of the cold agglutinin titers after the first penicillamine treatment (approximately 4 to 5 weeks) is contrasted by the rather rapid restoration of the titers following subsequent courses of penicillamine and mercaptopyridoxine. There is presently no satisfactory explanation for this discrepancy. Theoretically, three possibilities may be considered. The first possibility is that long-term treatment with relatively high dosages of mercaptanes may produce complete or almost complete "saturation" of the macroglobulins with sulfhydryl groups in both the circulation and the tissues. After cessation of treatment, restoration of the initial high concentrations of cold agglutinins may thus be delayed. In short-term treatment tissue macroglobulins may not be affected. Thereafter, influx of tissue antibodies into the circulation and continued formation of cold agglutinins may produce rapid restoration of the titers to treatment levels. The second possibility is that the sensitivity of macroglobulins to mercaptanes may be decreased and thus be reflected by a small decrease in the titer, with rapid restoration of the pretreatment concentration of cold agglutinin. Since the decrease in the titers in

the latter experiments was almost comparable to that in the previous tests, the latter suggestion is improbable. Third, the rate of metabolism of the administered mercaptanes may be accelerated. This possibility of "adaptation" of the organism cannot be ruled out at present. To prevent inactivation, mercaptanes should be given orally 2 hours before breakfast and 2 hours after the evening meal.

During the treatment with mercaptanes the reduction of the thermal amplitude from 32° or 30° to less than 25° C. in this patient is of considerable significance, since the titers (concentration) of the cold agglutinins determine the temperature range in which hemagglutination and hemolysis may be expected to occur (Figs. 9 and 10). Complete abolition of cold agglutinins is unnecessary since a marked and sustained reduction of the cold agglutinin titer would be sufficient to produce clinical improvement. As a result, the titers of cold agglutinins must be lower in cold climates than in warm climates if agglutination, hemolysis, and associated manifestations are to be averted.

Since cold agglutinin disease is a chronic unremitting disorder, long-term treatment or seasonal (winter period) reduction of cold agglutinin titers is the obvious goal. Intermittent long-term treatment with mercaptanes with relatively high therapeutic indices may be indicated in the presence of severe cold agglutinin disease.

D,l-Penicillamine has been administered over a period of several years to patients with hepatocerebellar degeneration (Wilson's disease)[25] without obvious side effects. However, toxic side effects, such as inhibition of growth in animals,[26] interference with vitamin B_6 utilization,[27] and with the activity of some liver transaminases,[28] as well as hematologic and dermatologic manifestations[29] have been produced by the L-form of penicillamine. D-Penicillamine appears to be nontoxic.[26] The high price of this compound, however, limits its liberal clinical use. Penicillin G potassium (with an unusually high therapeutic index) is partly metabolized into D-penicillamine[11, 30, 38] The effectiveness of penicillin G (given in dosages of 10 million units intravenously) in conjunction with probenecid has been demonstrated in a case of macrocryogelglobulinemia of Waldenström.[23, 31] However, its in vivo effectiveness in cold agglutinin disease has yet to be proved. Cysteamine, which is a constituent of coenzyme A, is a relatively unstable compound and has to be protected from decay by 100 per cent nitrogen atmosphere. The handling of sterile cysteamine for injection is technically difficult. Nevertheless, cysteamine has been administered intravenously in a few cases of macroglobulinemia of Waldenström.[24] Mercaptopyridoxine appears to be relatively safe if administered orally (250 to 500 mg. four times a day) or intravenously (0.3 Gm. twice or three times a day). A possible antimetabolic vitamin B_6 effect could be prevented by the simultaneous oral administration of vitamin B_6 (10 to 50 mg. per day). No toxic effects were observed during mercaptane therapy.

In view of the lack of effective therapeutic measure in cold agglutinin disease, the administration of certain mercaptanes may be helpful, and deserves further study.

SUMMARY

1. The effects of a number of mercaptanes upon a cold agglutinin were studied. The cold agglutinin was characterized by γ_1-β_2 position, 15.5 S_{20}, and high carbohydrate content.

2. Penicillin G potassium, d,l-penicillamine, cysteamine, and mercaptopyridoxine produced reduction of cold agglutinin titers in vitro. Penicillamine reduced the quantity of macromolecular cold agglutinins in the ultracentrifuge and resulted in transient changes of the cold agglutinin fraction in the electroserogram.

3. Administration of d,l-penicillamine and mercaptopyridoxine to the patient produced marked reduction in the titers of cold agglutinins and acid-cold hemolysins; the thermal amplitude of cold agglutinin activity was reduced and the Cr^{51} survival curve of erythrocytes was changed, which suggested lengthening of the survival time of the erythrocytes. The patient's general condition was improved.

4. Depolymerization and inactivation of the macromolecular cold agglutinins by the mercaptanes appears to be the underlying mechanism.

5. These reproducible results with mercaptanes seem to offer an effective therapeutic approach to cold agglutinin disease.

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STUDIES ON THE DESTRUCTION OF GLUTATHIONE-UNSTABLE RED BLOOD CELLS

The Influence of Fava Beans and Primaquine Upon Such Cells in Vivo

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Red cell glutathione was decreased and unstable in an individual who had experienced acute hemolytic episodes after the ingestion of fava beans. The hematologic findings during such an episode in this individual differed from those observed in dogs during experimental oxidant-induced hemolysis in that red cell Heinz bodies and an elevated methemoglobin concentration in the blood were absent and the duration of hemoglobinemia was prolonged in the patient with favism. It was not possible to demonstrate any agglutination of red cells sensitized in vitro with fava bean extract by serum from the patient.

The survival of Cr^{51}-labeled glutathione-unstable red cells in normal human recipients was not altered when the recipients ate fava beans. The ingestion of primaquine by the recipients was, however, followed by rapid removal of these cells from the circulating blood. This removal was attended by an accumulation of radioactivity in the liver predominantly and in the spleen to a lesser extent.

The data indicate that: (1) Glutathione instability of red cells of persons with favism is not the sole factor in the hemolytic reaction to fava beans. (2) Glutathione-unstable red cells of persons with favism are sensitive to primaquine. (3) Hemolysis of glutathione-unstable red cells by daily doses of primaquine occurs predominantly intravascularly and the rate of hemolysis in vivo correlates with the degree of glutathione-instability of the red cells in vitro. Ascorbic acid may afford some protection against this intravascular lysis in vivo.

THE ingestion of fava beans by susceptible individuals results in a severe acute hemolytic reaction characterized by hemoglobinemia and hemoglobinuria.[1] The demonstration in several laboratories[2-7] that the red cells of patients with favism are deficient in glucose-6-phosphate dehydrogenase and hence are defective with respect to glutathione (GSH) stability has served to relate favism to the acute hemolytic anemia that occurs in sensitive individuals after the ingestion of drugs such as primaquine, the demonstrated red cell abnormalities being identical in both conditions. The hemolytic effect of primaquine is probably best explained on the basis of its metabolism to an oxidant

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substance such as a substituted 5-hydroxy-8-aminoquinoline in a manner analogous to the metabolism of 8-aminoquinoline itself to 5-hydroxy-8-aminoquinoline.[8] The latter compound causes methemoglobin production and lysis of normal red blood cells in vitro.[8] Red cells with unstable GSH are probably even more susceptible to lysis by such an oxidant substance than are normal red cells. However, neither the mechanism of the hemolytic reaction in favism nor the nature of the action of the fava bean or its derivatives on the GSH-unstable red cells is clear. The purpose of this paper is to present some observations made on a patient with favism and to compare them with observations made in experimental oxidant-induced hemolytic anemia. The influence of fava beans and of primaquine upon the survival in normal recipients of the GSH-unstable red cells of this patient and of those of an individual not sensitive to fava beans will also be described.

METHODS

Hematocrits, hemoglobin concentrations, mean corpuscular indices, icterus indices, reticulocyte counts, and other hematologic determinations were done by standard methods.[9] Plasma and urine hemoglobin concentrations were measured by a photoelectric modification of the benzidine method of Bing and Baker.[9]

Red blood cell survival curves and the sites of red blood cell sequestration after their removal from the circulation were determined by using radioactive chromium (Cr^{51}) according to the methods of Jandl and associates.[10] In all cases GSH-unstable red cells were labeled with $Na_2Cr^{51}O_4$ in vitro and injected into hematologically normal human recipients. One hundred milligrams of ascorbic acid was added to the red cells prior to injection to convert Cr^{51} to the trivalent form in order to prevent labeling of the recipient's own red cells. Fava beans and/or primaquine were subsequently administered to the recipients as indicated below.

Tests for the sickling phenomenon were done by the metabisulfite method.[11] Paper electrophoresis of stroma-free hemoglobin solutions was carried out in an apparatus described by Smith and Conley.[12] Wet preparations of blood were stained with methyl violet to show Heinz bodies, as described by Webster and co-workers.[13] Methemoglobin concentrations were measured by the method of Evelyn and Malloy,[14] and expressed as percentage of total hemoglobin.

The reduced GSH content of whole blood was measured on freshly drawn, heparinized venous blood by a modification of the method of Grunert and Phillips.[15] The stability of red cell GSH in the presence of acetylphenylhydrazine (APH) was determined by Beutler's method.[16] The range of values of GSH content of red cells determined on the bloods of twenty-four hematologically normal men and women was from 58.3 to 96.5 mg. per 100 ml. red blood cells; the range of the GSH content after the same bloods had been incubated in the presence of APH for two hours was from 45.9 to 85.4 mg. per 100 ml. red blood cells. These normal values agree closely with Beutler's data.

The Coombs test[17] was performed on saline-washed red cells with a potent commercial antihuman-serum rabbit serum. Red cells were also tested for the presence of adsorbed protein by means of the polyvinylpyrrolidone (PVP) test of Jandl and Castle.[18]

The agglutination of normal red cells and of red cells with unstable GSH by saline extracts of dried fava beans, and the effects of normal serum and of serum from the patient with favism upon such agglutination were studied. In some cases, red cells were treated with tannic acid according to Boyden's method[19] before being put into contact with the fava bean extract. It was hoped that by this means some of the fava bean protein could be adsorbed onto the red cells so that the latter might serve as indicators of an antigen-antibody reaction when serum from the patient with favism was added.

The saline extract of fava beans was prepared by blending 1 part (by weight) of dried beans with 4 parts of isotonic saline in a Waring Blendor. The suspension was then centrifuged at 2,000 r.p.m. for 30 minutes. The supernatant was filtered through Whatman No. 42 filter paper.

CLINICAL MATERIAL

The subject with favism was a 4-year-old boy of Greek descent who was hospitalized on two occasions because of hemoglobinuria following the ingestion of cooked dried fava beans. During each of these hemolytic episodes, when he was acutely ill, febrile, and icteric, the spleen was palpable. At other times the physical examination showed normal health. Severe anemia developed during each episode. The hemoglobin concentration fell to 3.9 Gm. per cent during the first and to 4.7 Gm. per cent during the second episode; small transfusions of packed red blood cells were given on each occasion. During the hemolytic episodes there was leukocytosis without abnormal or immature granulocytes in the peripheral blood; platelets were normal; reticulocyte counts were elevated; and there was marked hemoglobinemia. The Wright stained blood film revealed marked variation in size and shape of the red cells, with many fragmented cells, spherocytes, and polychromatophilic cells. The direct Coombs test was persistently negative, as was the PVP test for sensitized red cells. Sickling preparations were negative. Only hemoglobin A was detected by paper electrophoresis in hemolysates of the red cells. Heinz bodies were not found in wet preparations of the red cells stained with methyl violet. Less than 1 per cent of the total hemoglobin was methemoglobin.

The patient and his immediate family were studied 5 months after his second hemolytic episode. At this time blood was taken for the transfusion studies described below.

The laboratory results are indicated in Table I. The mother was in the second trimester of pregnancy, this was thought to be the cause of her mild anemia. The younger sister had a mild hypochromic anemia. It can be seen that the patient, his father, and his

TABLE I. HEMATOLOGIC FINDINGS IN THE FAMILY OF A PATIENT WITH FAVISM

SUBJECT	AGE IN YEARS	HEMOGLOBIN (GM./100 ML.)	HEMATOCRIT (%)	R.B.C. GSH (MG./100 ML.)	R.B.C. GSH AFTER INCUBATION WITH APH* (MG./100 ML.)
Patient	5	12.6	39	45.3	4.3
Sister	11	12.0	38	59.6	8.7
Sister	2	10.9	37	44.0	
Mother†	34	10.7	31	52.8	32.7
Father	34	15.1	49	81.8	71.7

*Blood was incubated with acetylphenylhydrazine (APH) according to Beutler's test for determining glutathione (GSH) instability.[16]

†Mother was in second trimester of pregnancy.

older sister were all normal by the routine hematologic tests at that time. However, the three children all had abnormally low red cell glutathione levels. Also, the glutathione stability of their red cells in the presence of APH was defective. In these respects the father was completely normal; the mother, however, was intermediate. The father frequently ate fava beans but had never had any reaction to the beans nor had he ever had an acute hemolytic episode. The mother never ate the beans and she knew of no relatives who were sensitive or who had ever had an acute hemolytic episode. The older sister of the patient frequently ate fava beans but had never had any reaction to them, hemolytic or otherwise. The mother did not know definitely whether or not the younger sister had ever eaten the beans, but knew that neither of the girls had ever had red urine.

The second donor used in the transfusion experiments was a 20-year-old Negro woman in good health, whose red cells evidenced impaired ability to maintain GSH in the reduced form in the presence of acetylphenylhydrazine. She had never had a hemolytic reaction, nor had she ever noted red urine. There was no history of jaundice or allergic reactions. Physical examination showed normal health.

Laboratory examinations revealed hemoglobin 10.2 Gm. per cent, hematocrit, 34 per cent. The sickling test was negative and only hemoglobin A was present in hemolysates of the red cells. Red cell GSH level was 56.9 mg. per 100 ml. red blood cells, and this fell to 13.4 mg. per 100 ml. red blood cells after incubation for 2 hours with acetylphenylhydrazine.

On several occasions blood was taken from this subject, labeled with $Na_2 Cr^{51} O_4$, and injected into hematologically normal persons. Details of these experiments will be described.

The recipients were volunteers who were hematologically normal. Their red cells were normal with regard to GSH concentration and stability in the presence of acetylphenylhydrazine.

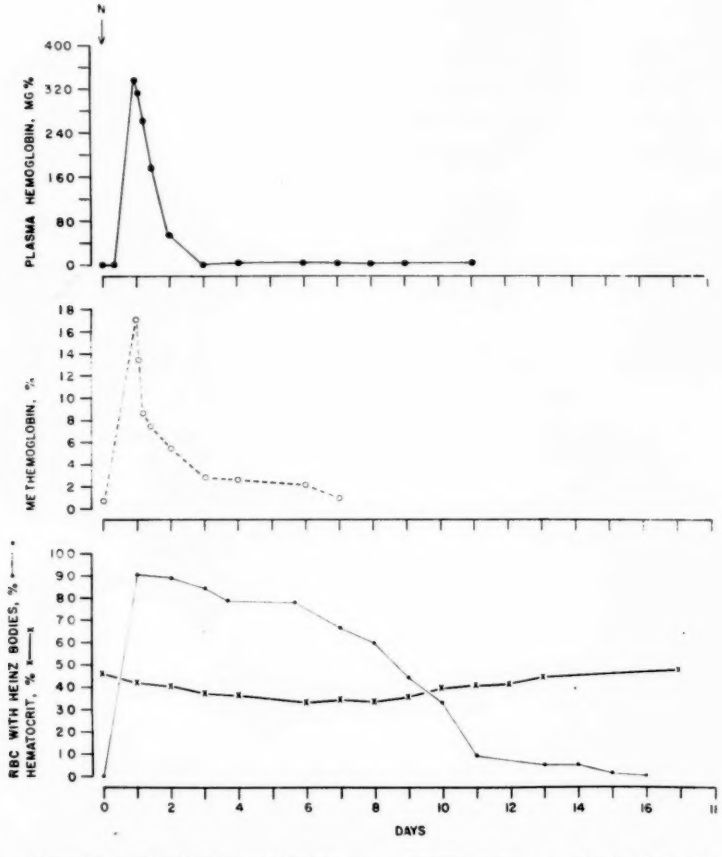

Fig. 1.—Hemolytic effect of naphthalene in a normal dog. A single dose of naphthalene (0.66 Gm. per kilogram body weight) was given on Day zero as indicated by the arrow at the top of the chart.

RESULTS

A. Comparison of the Changes Observed in the Blood During Acute Hemolytic Anemia Due to Fava Bean Ingestion With Those Observed in Experimental, Oxidant-Induced, Acute Hemolytic Anemia.—Sixteen grams (0.66 Gm. per kilogram) of naphthalene dissolved in corn oil was administered through a gastric tube to a normal dog. An acute hemolytic anemia characterized by methemoglobinemia, hemoglobinemia, hemoglobinuria, and Heinz

body formation ensued. The sequence of the hematologic changes that occurred in the dog are depicted in Fig. 1, and may be compared with the observations made on the subject with favism during the course of one of his acute hemolytic episodes (Fig. 2). While the data in the case of our patient are of necessity not complete, some comparisons between the changes in the patient and those in the dog are of interest.

1. The rate of development of anemia appears to be the same, the maximum degree of anemia occurring about 6 days after the administration of the toxic agent.

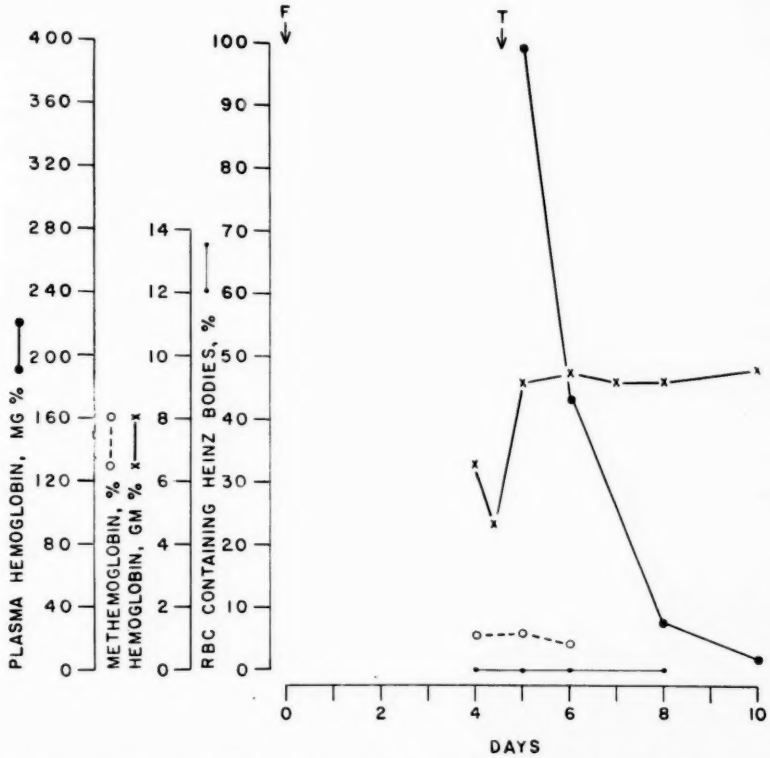

Fig. 2.—Hemolytic effect of fava beans in a fava-sensitive human being. The subject had eaten a few spoonfuls of cooked dried fava beans on Day zero as indicated by the arrow marked *F*. On the fourth day a transfusion of 200 ml. of packed red blood cells was given. Note that no Heinz bodies were present in the peripheral blood on the fourth, fifth, sixth, and eighth days.

2. In the case of the dog, methemoglobin and plasma hemoglobin concentrations were maximal at 24 hours and then fell rapidly, reaching normal values by the third day. Heinz bodies were not present in the circulating red cells 10 hours after the administration of naphthalene but were present in maximal concentration at 24 hours. Thereafter, the number of red cells containing Heinz bodies fell gradually over a period of about 2 weeks, and the Heinz bodies did not completely disappear from the blood until the fifteenth or sixteenth day. Their rate of removal paralleled the fall of circulating hemoglobin for the first 6 days. After that time the increased production of

reticulocytes prevented a further decrease in circulating hemoglobin concentration, even though destruction of red cells containing Heinz bodies continued at a rate greater than that of the destruction of normal red cells. Thus, when the circulating hemoglobin concentration reached its nadir on the sixth day, the methemoglobin and plasma hemoglobin concentrations were normal, though a significant number of red cells still contained Heinz bodies.

3. In the case of the patient with favism, the first observations were made 24 hours before the circulating hemoglobin concentration reached its lowest level. At this time and subsequently, Heinz bodies were not present in the red cells and there was no elevation of the concentration of methemoglobin. However, the plasma hemoglobin concentration was markedly elevated.

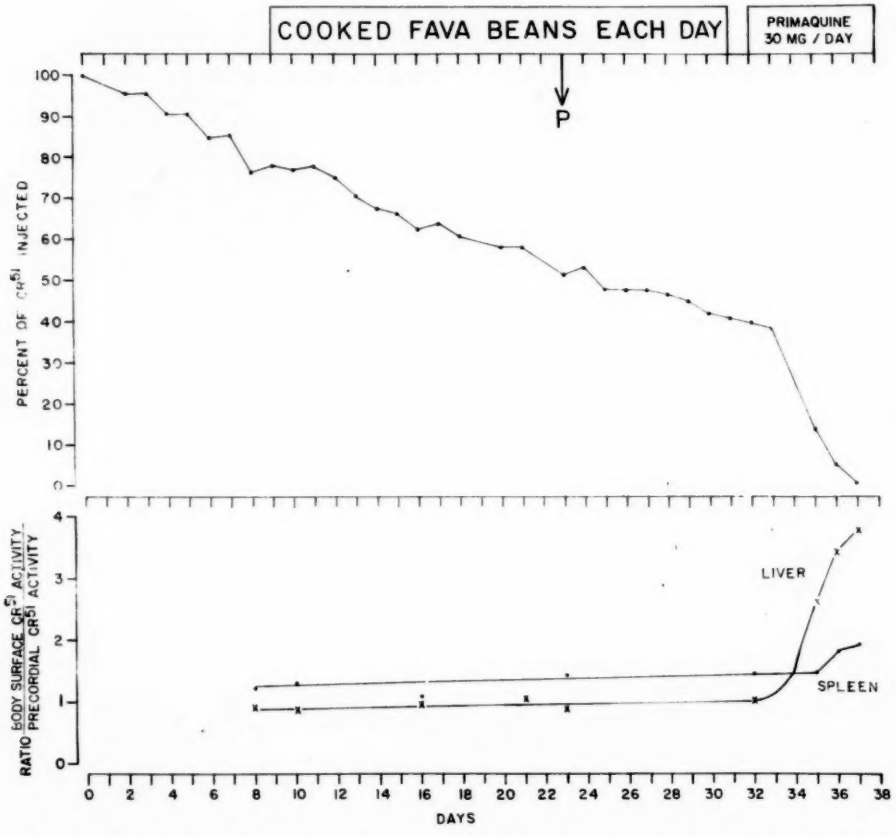

Fig. 3.—Survival and organ sequestration of Cr^{51}-labeled GSH-unstable red blood cells from a subject sensitive to fava beans in a normal human recipient. On the twenty-third day 30 ml. of fresh plasma from the same donor was given intravenously to the recipient. The biologic half-life of the labeled cells was 25 days during the control and fava bean periods. However, rapid removal of the labeled cells from the circulation occurred after the administration of primaquine, the half-life then being 2.2 days. There was no significant change in hematocrit throughout the study, indicating that the recipient's own red cells were unaffected by either the fava beans or the primaquine.

B. *The Effect of Fava Bean Ingestion Upon Survival of GSH-Unstable Red Cells in Hematologically Normal Recipients.*—Thirty to forty milliliters of blood from the normal subject known to have GSH-unstable red cells was

labeled with $Na_2Cr^{51}O_4$ in vitro and injected into each of two hematologically normal recipients. After a brief control period for estimation of the survival time of the cells in each recipient, one recipient was fed several portions of cooked dried fava beans each day for a 10 day period, and the other was given 15 mg. of primaquine twice each day. Neither recipient developed any significant change in hematocrit. The transfused labeled GSH-unstable red cells rapidly disappeared from the peripheral blood of the subject treated with primaquine, while the ingestion of fava beans had no effect upon the survival of similar red cells in the other recipient.

The same experiment was then repeated, with the use of GSH-unstable red cells taken from the patient with favism about 5 months after his most recent acute hemolytic episode. The results were similar. As anticipated, there was rapid removal of the labeled red cells from the circulation in a recipient who was given primaquine. The ingestion of fava beans for a period of 14 days had no effect upon the survival of the labeled red cells in another recipient (Fig. 3). Thirty milliliters of plasma freshly drawn from the patient with favism was infused intravenously into this latter recipient on the fourteenth day. The ingestion of fava beans was continued for another 8 days, again without effect on the survival of the labeled red cells. The subsequent administration of primaquine to this subject was attended by rapid disappearance of the labeled red cells from the circulation, indicating that the red cells were truly "primaquine sensitive" in this particular recipient.

It is of some interest to note that the rate of removal of these two populations of GSH-unstable red cells from the circulation correlates with the stability of their GSH. GSH concentrations before and after incubation of the cells with APH for the red cells of the patient with favism (Donor I) and for those of Donor II, are given in Table II, together with the in vivo half-lives of these cells in several recipients before and after administration of primaquine in daily oral doses to the recipients. Although the degree of aging in vivo of the transfused cells prior to the administration of primaquine to the recipients must be considered in the interpretation of the hemolytic effect of primaquine,[20, 21] no difference was observed between the rate of hemolysis of Donor I cells in the recipient who was given primaquine 32 days after transfusion (Fig. 3) and the rate in the recipient who was given primaquine 9 days after transfusion. In each recipient the labeled Donor I red cells disappeared from the circulation with a half-life of 2.2 days. On the other hand, the postprimaquine half-life of the Donor II cells in recipients started on primaquine 11 and 13 days after transfusion was 5.4 days in the first recipient and 5.0 days in the second. Further, in each recipient of Donor I cells more than 90 per cent of the transfused cells had disappeared from the circulation within 8 days of the onset of primaquine ingestion. In contrast, about 30 per cent of the transfused Donor II cells seemed resistant to lysis by primaquine in each of the two recipients of these cells (Fig. 4).

The half-lives of the cells of both donors prior to the administration of primaquine to the recipients were similar (Table II) and close to the normal range of 28 to 33 days.

TABLE. II. SURVIVAL OF Cr^{51}-LABELED GSH-UNSTABLE RED BLOOD CELLS IN NORMAL HUMAN RECIPIENTS

R.B.C. GSH (MG./100 ML.)	R.B.C. GSH AFTER INCUBATION WITH APH (MG./100 ML.)	RECIPIENT	HALF-LIFE BEFORE PRIMAQUINE* (DAYS)	TIME AFTER TRANSFUSION† BEFORE PRIMAQUINE (DAYS)	HALF-LIFE WITH PRIMAQUINE‡ (DAYS)	PROPORTION OF LABELED R.B.C. RESISTANT TO PRIMAQUINE§ (%)
Donor I R.B.C. (fava sensitive).—						
45.3	4.3	1	25	9	2.2	7.8
		2	25	32	2.2	0
Donor II R.B.C.—						
56.9	13.4	3	26	11	5.4	28.8
		4	24	—	—	—
		5	27	13	5.0	29.3
		6‖	25	8	7.0‖	37.4‖

*In vivo half-life of the labeled R.B.C. prior to the administration of primaquine to the recipients.

†In vivo aging of the labeled R.B.C. in the recipients prior to the administration of primaquine.

‡In vivo half-life of the labeled R.B.C. during the administration of primaquine to the recipients.

§The number of labeled R.B.C. surviving in the circu'ation after daily oral ingestion of primaquine by the recipients for 8 days, expressed as percentage of the total number of transfused labeled cells.

‖Recipient No. 6 was given ascorbic acid orally (1.2 Gm. per day) during the time he was taking primaquine.

C. Sites of Sequestration of Cr^{51}-Labeled Transfused GSH-Unstable Red Cells During Their Rapid Disappearance From the Circulating Blood After the Administration of Primaquine to the Recipients.—The lower portion of Fig. 3 depicts the changes in the ratios of liver and spleen radioactivity to precordial radioactivity, as determined by body surface counting over these organs in the recipient transfused with Cr^{51}-labeled GSH-unstable red cells from the patient with favism. Radioactivity did not accumulate significantly in the liver or spleen until primaquine was given. Then, as the radioactive, labeled red cells were removed from the circulating blood, the radioactivity accumulated predominantly in the liver.

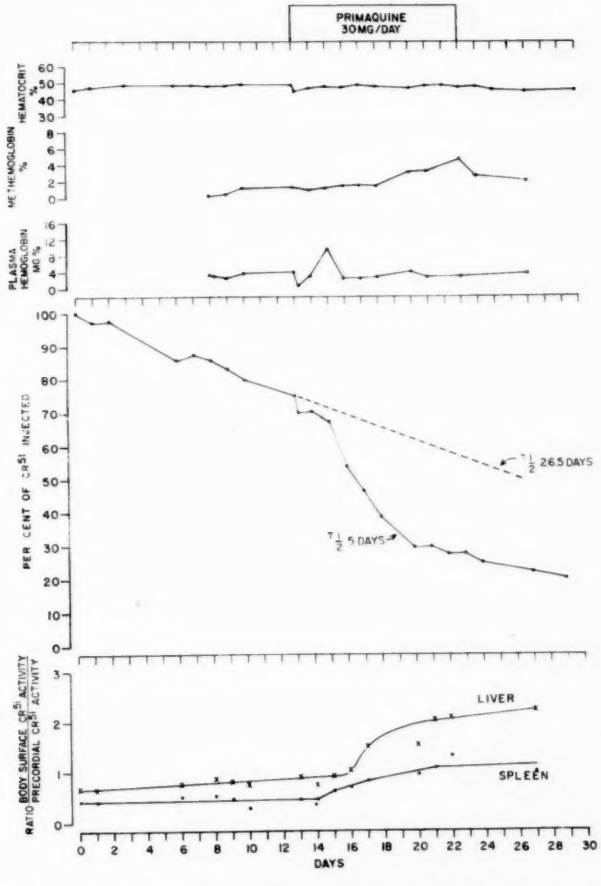

Fig. 4.—The hemolysis of GSH-unstable red blood cells by primaquine in vivo.

Fig. 4 indicates that similar, though less marked, accumulation of radioactivity occurred in the liver when a recipient of GSH-deficient red blood cells from the other donor was given primaquine.

D. The Effect of Ascorbic Acid Upon the Survival of GSH-Deficient Red Cells in Normal Recipients Taking Primaquine.—The simultaneous daily oral

administration of 0.4 Gm. of ascorbic acid and 30 mg. of primaquine had no observable effect upon the survival time of Cr^{51}-labeled GSH-deficient red cells derived from the patient with favism, as compared with effect upon the survival of the same red cells in another normal recipient taking primaquine only. However, the daily oral administration of 1.2 Gm. of ascorbic acid together with primaquine, to a recipient of Donor II GSH-unstable red cells may have had a beneficial effect upon the survival of these cells. Thus, the half-life of these cells in the recipient taking ascorbic acid was 7 days in contrast to 5 days in the other subjects, and a slightly greater proportion of the labeled red blood cells survived 8 days of primaquine (Table II).

E. Effect of Saline Extracts of Dried Fava Beans Upon Red Cells in Vitro.—Incubation of normal and GSH-unstable red cells with the saline extract of dried fava beans caused neither Heinz body formation, methemoglobin production, nor hemolysis of the red cells. Nevertheless, the fava bean extract did cause marked agglutination of normal and GSH-unstable red cells suspended in saline. GSH-unstable red cells were not more sensitive than normal red cells to agglutination by the fava bean extracts. In each case suspension of the cells in the bean extract diluted 1:80 with saline resulted in agglutination. In higher dilutions of the bean extract agglutination did not occur. However, red cells suspended in dilutions of bean extract as great as 1:2,560 were agglutinated when suspended in either a 10 per cent solution of acacia or a 5 per cent solution of PVP. In these experiments the cell suspensions in diluted fava bean extract were incubated at 37° C. for 30 minutes and then washed 3 times in isotonic saline. The washed packed cells were then suspended in an equal volume of either 10 per cent acacia or 5 per cent PVP. In both colloidal solutions agglutination of the cells occurred. Again no differences were observed between the susceptibility to agglutination of GSH-unstable red cells and that of normal cells.

F. Effect of Serum From the Patient With Favism Upon Red Cells Sensitized With Fava Bean Extract.—Normal serum inhibited the agglutination of both normal and GSH-unstable red cells by the saline extract of fava beans even when the serum was present in great dilution (1:250). Serum obtained from the patient during the course of his second hemolytic episode ("favism serum") similarly inhibited agglutination.

Normal and GSH-unstable red cells were sensitized by incubation in a 1:100 dilution of the fava bean extract for 30 minutes at 37° C. The red cells were washed 3 times and resuspended in a 1:250 dilution of normal human serum in saline. The addition of "favism serum" did not cause agglutination. The cells were washed 3 times again in isotonic saline. No agglutination resulted when Coombs serum was added to the washed packed red cells. The same reactions occurred when normal human serum was used in place of "favism serum." Similar reactions were also observed when the red cells were suspended in a 1:20,000 solution of tannic acid for 10 minutes at 37° C.[19] and then washed 3 times in isotonic saline immediately before being suspended in the bean extract.

DISCUSSION

That the red blood cells of an individual with favism are glutathione-unstable and that these cells are, in fact, also sensitive to hemolysis by primaquine is confirmed by the present data. However, some critical differences between the mechanism of hemolysis in favism and that in oxidant-induced hemolytic anemia have been demonstrated. The observation that the sister of the subject with favism did not develop acute hemolytic anemia on many occasions after the ingestion of fava beans, although her red cell glutathione concentration and stability were comparable to those of her fava bean–sensitive brother, suggested that a factor other than red cell glutathione instability is involved in favism. This concept is further supported by the failure of fava bean ingestion to alter the survival of glutathione-unstable red cells in normal recipients. What this other factor is has not been demonstrated. Following are some of the alternate possibilities:

(1) Unusual metabolism of fava beans with the production of a metabolite which acts as an oxidant and affects glutathione-unstable red cells in a manner similar to that of primaquine derivatives.

(2) Unusual metabolism of fava beans with the formation of a derivative of the bean having lytic properties only against glutathione-unstable red cells but acting in a manner other than as an oxidizing agent.

(3) Usual metabolism of fava beans with the formation of a metabolite against which are produced antibodies which have lytic effects on glutathione-unstable red cells.

That a derivative of the fava beans which has oxidant properties and acts like a metabolite of primaquine or of naphthalene appears in the plasma of patients with favism seems unlikely in view of the differences noted in Figs. 1 and 2 between the hemolytic anemia in the patient with favism and that caused by naphthalene. Thus, the persistence of hemoglobinemia and hemoglobinuria 6 days after the ingestion of the fava beans and the absence of Heinz bodies from the red cells at the time when the circulating hemoglobin level was falling suggest that the mechanism of hemolysis in the reaction to fava beans was not due to the presence of an oxidizing agent. Others have also reported the absence of Heinz bodies in the red cells during acute hemolytic episodes due to favism.[3] The observations of Beutler and associates[22] that Heinz bodies do appear in the red cells of sensitive individuals in whom hemolytic anemia is produced by the administration of small doses of primaquine would suggest that the mechanism of hemolysis in such cases is similar to that produced in normal dogs by the administration of large amounts of naphthalene. The normal methemoglobin concentration of the blood during the course of the hemolytic reaction to fava beans is a less convincing bit of evidence against the presence of an oxidant substance in this situation, since, in the case of the dog experiment, there was no significant elevation of methemoglobin concentration 6 days after the oral ingestion of a single dose of naphthalene. Methemoglobin production does not correlate directly with increased hemolysis

but if congenital methemoglobinemia is excluded, an increased amount of methemoglobin after the administration of drugs probably does indicate the presence of an oxidant substance in the circulating blood.

Creger and Gifford[23] demonstrated, and we have confirmed, that there are definite interactions in vitro between human red cells and saline extracts of fava beans. The bean extracts cause agglutination of the red cells; it is possible that such agglutination occurring in vivo may be of significance in the mechanism of hemolysis in favism. However, no difference between normal human and GSH-unstable red cells, in respect to agglutination by bean extracts, potentiation of such agglutination by colloids, or its inhibition by normal human serum or by the serum of the patient himself could be demonstrated. It would therefore seem that the agglutinin present in saline extracts of fava beans and active in vitro is not responsible for the hemolytic activity of fava beans in vivo.

An immune mechanism with the production of antibodies directed against red cells is an attractive hypothesis because of the small amounts of fava bean substance which may be effective in vivo. Luisada[24] indicates that as little as one bean, or in fact, merely the inhalation of pollen, may initiate an episode of hemolysis in a sensitive individual. In addition, incomplete warm agglutinins have been demonstrated in the sera of patients by means of direct and indirect Coombs tests during the hemolytic episode. Marcolongo[25] found such agglutinins in more than 80 per cent of 120 cases. Others have reported the transient presence of hemolysins.[26] In the case under discussion, these tests were negative. The observations by Vullo and Panizon[27] of a hemolytic effect in vivo of fava beans on Cr^{51}-labeled GSH-unstable red cells in 2 of 10 normal recipients further support the concept of an immunologic mechanism active only against GSH-unstable red cells.

The sequence of events in the anemia that followed the administration of a single dose of naphthalene to a normal dog began with appearance of oxidizing substances (alpha and beta naphthols) in the plasma with simultaneous intravascular lysis of a small number of red cells occurring about 12 to 24 hours after the naphthalene was given. At this time methemoglobin was also present and almost all of the red cells contained Heinz bodies. The blood methemoglobin and the plasma hemoglobin levels rapidly reverted to normal. The red cells containing Heinz bodies, on the other hand, disappeared slowly from the circulating blood over a period of about 15 days, as the anemia became more and more marked.

A similar sequence of events follows the administration of acetylphenylhydrazine to normal dogs.[28] Jandl and Tomlinson[29] have shown that red cells, labeled with Cr^{51}, treated in vitro with an oxidizing agent to form Heinz bodies, and transfused into a normal recipient, are sequestered gradually in the spleen over a period of about one week, confirming the prior demonstration by Cruz[30] that Heinz bodies appear in the spleen of normal dogs poisoned with acetylphenylhydrazine. Thus, it would appear that red cells are altered in several ways during the time when an oxidant substance is present in the plasma. Some, but relatively few, of the red cells are lysed intravascularly, with prompt release of

their hemoglobin into the plasma. A much greater proportion of red cells is damaged in a manner that causes the formation of Heinz inclusion bodies. These red cells are subsequently sequestered gradually in the spleen over a period of days, and their disappearance from the circulating blood is associated with progressive anemia. During the time when the oxidant substance is present in the plasma, methemoglobin can be detected in the red cells. This, however, bears no stoichiometric relationship to hemolysis, since methemoglobinemia may develop in individuals given drugs such as sulfanilamide and pamaquine, even though no apparent increased rate of hemolysis occurs. The methemoglobin and elevated plasma hemoglobin rapidly disappear after the oxidant substance is cleared from the plasma.

In the present study it was found that when primaquine was administered daily to recipients of Cr^{51}-labeled GSH-deficient red cells, radioactivity accumulated predominantly in the liver as the labeled cells disappeared from the circulation. It was also noted (Fig. 4) that there was a small elevation in the concentration of plasma hemoglobin during the time when the labeled cells were disappearing from the circulation. It has been previously observed[10] that, after the intravenous injection of a solution of Cr^{51}-labeled hemoglobin, prepared by lysing Cr^{51}-labeled red cells in vitro, radioactivity accumulated predominantly in the liver as it disappeared from the circulation. These data suggest that, when primaquine is given daily, the presence of an oxidant substance in the plasma is prolonged and that the predominant mechanism of hemolysis of susceptible red cells is direct intravascular lysis. It is of interest to note that in these recipients the concentration of methemoglobin slowly rose in the recipients' own normal red cells during administration of the drug even though anemia did not occur. The administration of ascorbic acid (1.2 Gm. per day) did not prevent an increased rate of destruction of labeled GSH-unstable red cells. However, the survival of such cells was improved slightly (Table II). This would suggest that the presence of a reducing agent in the plasma may interfere with the action of an oxidant substance upon GSH-unstable red cells. Factors such as this might account, in part, for the differences noted by Dern and associates[21] in the survival of Cr^{51}-labeled GSH-unstable red cells from a single donor in different recipients taking thiazolsulfone.

The observed correlation between glutathione concentration and stability of red cells and their in vivo survival in the presence of primaquine supports the concept that either glutathione itself is necessary for the maintenance of red blood cell integrity or its concentration is a manifestation of another red cell factor which is essential in this respect.

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ALKALINE PHOSPHATASE ACTIVITY OF THE POLYMORPHONUCLEAR LEUKOCYTE IN RAPIDLY INDUCED LEUKOPENIA AND LEUKOCYTOSIS

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The changes in unit alkaline phosphatase activity of human polymorphonuclear leukocytes have been studied in rapidly induced leukopenic and hyperleukocytic states. The subjects were divided into four groups: I. Pyrexal (Salmonella lipopolysaccharide), 0.1 μg, injected intravenously; II. Epinephrine, 1 ml. of 1:1,000, injected intramuscularly; III. Saline control, 2 ml. normal saline; IV. Prednisolone, 40 μg, injected intravenously. At intervals of 0, 15, 30, 60, 90, and 120 minutes after injection, total white blood cell and polymorphonuclear cell counts were performed. Simultaneously, the leukocytes were separated and assayed for alkaline phosphatase. Thirty minutes after Pyrexal injection the leukocyte count dropped to approximately 25 per cent below pretreatment levels. The unit alkaline phosphatase activity of the polymorphonuclear cells dropped to about 30 per cent of pretreatment values. Sixty minutes after epinephrine injection a 35 per cent rise in leukocyte count was observed. Phosphatase activity paralleled this change, increasing about 50 per cent above pretreatment level. No significant change was observed in either alkaline phosphatase activity or leukocyte count in the saline control group. In contrast to epinephrine or pyrogen, prednisolone produced no change in cell count over the course of several hours. The unit alkaline phosphatase activity, however, dropped within 15 minutes to about 40 per cent of pretreatment levels and then returned to initial values in 45 minutes.

THE leukocytes in the circulating blood stream are capable of marked and rapid shifts in number. The direction of the change of the peripheral leukocyte count following a given stimulus remains fairly constant. Thus, pyrogens invariably trigger a leukopenia, whereas leukocytosis follows the administration of epinephrine.[1-5] The alkaline phosphatase activity of the leukocyte has been found to be quite labile.[6, 7] Results of previous studies from this laboratory[8, 9] have been consistent with the hypothesis that alkaline phosphatase activity may serve as a measure of the physiologic maturity of the polymorphonuclear leukocyte. It seemed logical, therefore, to study the changes in unit alkaline phosphatase activity of the polymorphonuclear cells in the leukopenia which was induced by pyrogens, and in the leukocytosis provoked by epinephrine, in an attempt to gain further information on the mechanisms

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of these responses. The data to be presented suggest that the rapid shifts in the number of circulating leukocytes are due to selective removal or delivery of polymorphonuclear cells from or into the blood stream. The action of steroids was similarly studied.

MATERIALS AND METHODS

*Group I: Pyrexal.**—Twelve patients were injected intravenously with 0.1 μg of Pyrexal in 2 ml. of saline.

Group II: Epinephrine.—Seven patients were injected intramuscularly with 1 ml. of 1:1,000 epinephrine (U.S.P.).

Group III: Saline Controls.—Four patients were injected intramuscularly with 2 ml. of normal saline.

Group IV: Prednisolone.†—Twelve patients were injected intravenously with 40 μg of prednisolone in 2 ml. of saline.

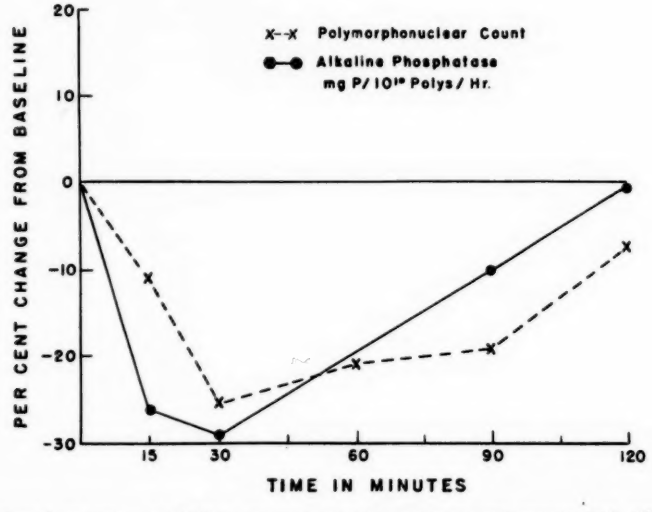

Fig. 1.—Pyrexal group: variation in polymorphonuclear cell count and alkaline phosphatase after administration of 0.1 γ Pyrexal intravenously.

At intervals of 0, 15, 30, 60, 90, and 120 minutes after the injection, a sample of peripheral blood was obtained and total white blood cell and polymorphonuclear cell counts (i.e., all segmented and band forms) were performed. Simultaneously, and immediately after collection, the white blood cells were separated, washed, and counted; the alkaline phosphatase content of the polymorphonuclear cells was then determined. Details of the separation procedure and of the determinations of alkaline phosphatase activity have been previously described.[8] After the administration of Pyrexal or epinephrine no unusual clumping of the white blood cells was observed, and no difficulty was encountered in the performance of the leukocyte counts of the whole blood or of the separated white blood cells.

Enzyme activity was expressed as milligrams of phosphorus released from the substrate (beta-glycerophosphate) in 60 minutes at 37° C. by 10^{10} polymorphonuclear leukocytes (mg. P/10^{10} polymorphonuclear leukocytes per hour).

*Pyrexal, Salmonella lipopolysaccharide, prepared by the Wander Company, Chicago, Ill., and supplied by Dr. Fred H. Shultz, Jr., Director of Research.

†Prednisolone, American Quinine Company, New York, N. Y.

RESULTS

The data in the accompanying graphs are expressed as per cent change from the base-line pretreatment level. The total count and the unit alkaline phosphatase activity in any single observation shown represent the mean value for the group.

Group I: Pyrexal-Induced Leukopenia.—Fig. 1 shows the results obtained in twelve male patients who were given 0.1 γ of Pyrexal intravenously. In all cases small febrile responses were noted. Mild headache and generalized aches accompanied the elevated temperature, but both symptoms and fever subsided rapidly and no untoward effects were noted. In all instances a drop in the white blood cell count was found, due chiefly to a decrease in the polymorphonuclear cells. The fall in leukocytes began very soon after the administration of the pyrogen, and reached its nadir, at approximately 25 per cent below pretreatment levels, in about 30 minutes, much before the appearance of the febrile reaction. The leukocyte count remained fairly constant for the next hour and then slowly returned to pretreatment levels in about 120 minutes.

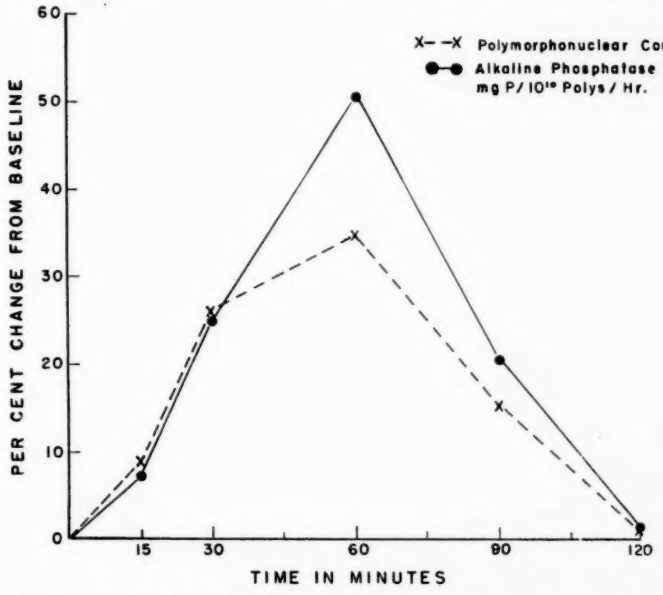

Fig. 2.—Epinephrine group: variation in polymorphonuclear cell count and alkaline phosphatase after administration of 1 ml. of 1:1,000 epinephrine intramuscularly.

Again, in all subjects, the unit alkaline phosphatase activity of the polymorphonuclear cells dropped after the injection of Pyrexal, to about 30 per cent of pretreatment values. The fall in alkaline phosphatase activity was greater than that of the leukocyte count. Thus, at the end of 15 minutes, the cell count had decreased to about 12 per cent of pretreatment levels, while the alkaline phosphatase activity was some 25 per cent below initial values. On the other hand, the phosphatase activity showed an earlier return to the pretreatment level.

It appears, therefore, that following the administration of a pyrogenic substance, the granulocytes of higher alkaline phosphatase content leave the blood stream preferentially to those of lower phosphatase activity. Conversely, the cells of higher phosphatase activity return more rapidly from sequestration sites into the circulating blood.

Group II: Epinephrine-Induced Leukocytosis.—In the experiments carried out in the present study, approximately a 35 per cent rise in white blood cell count was observed about 60 minutes after the intramuscular injection of 1 ml. of 1:1,000 epinephrine. The results (Fig. 2) indicate that the rise of the white blood cell count was prompt and returned to pretreatment values in 120 minutes. The phosphatase activity paralleled the change in leukocyte number, reaching its maximum of about 50 per cent above pretreatment values in 1 hour, coincidentally with the height of the increase in cell count. The phosphatase activity fell to pretreatment levels in 120 minutes.

The leukocytosis induced by epinephrine may be best accounted for by a selective flow of enzyme-rich cells into the peripheral blood stream and a prompt departure of these cells from the circulation with the subsidence of the reaction.

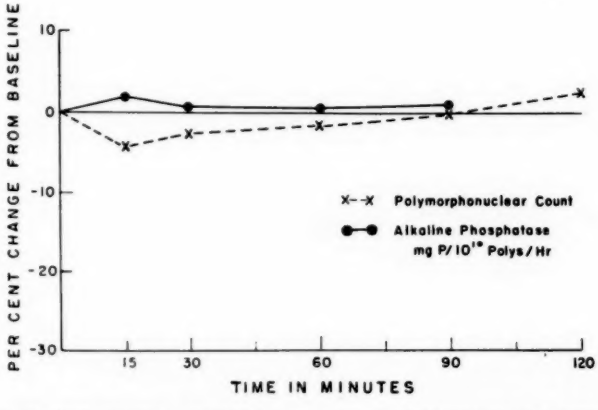

Fig. 3.—Saline control group: variation in polymorphonuclear cell count and alkaline phosphatase after administration of 1 ml. of physiologic saline intramuscularly.

Group III: Saline Control.—The pattern of cell count and alkaline phosphatase activity after the intramuscular administration of physiologic saline is given in Fig. 3. It is clear that no significant change in either parameter was observed.

Group IV: Response to Prednisolone.—The behavior of the leukocyte count and the enzyme activity following the intravenous administration of Prednisolone is given in Fig. 4. These experiments were initiated to evaluate the possible role of the steroids in mediation of the changes observed with the pyrogen or epinephrine. In contrast to epinephrine or pyrogen, prednisolone produced no change in cell count throughout the entire 120 minutes of observation. The unit alkaline phosphatase activity of the polymorphonuclear cell, however, showed a bidirectional change during the experimental period.

Within 15 minutes there was a rapid drop of phosphatase activity of about 40 per cent from pretreatment levels, followed by a prompt return to baseline values in 45 minutes. Then a steady rise in activity to about 25 per cent above the pretreatment level was noted, and no further change occurred.

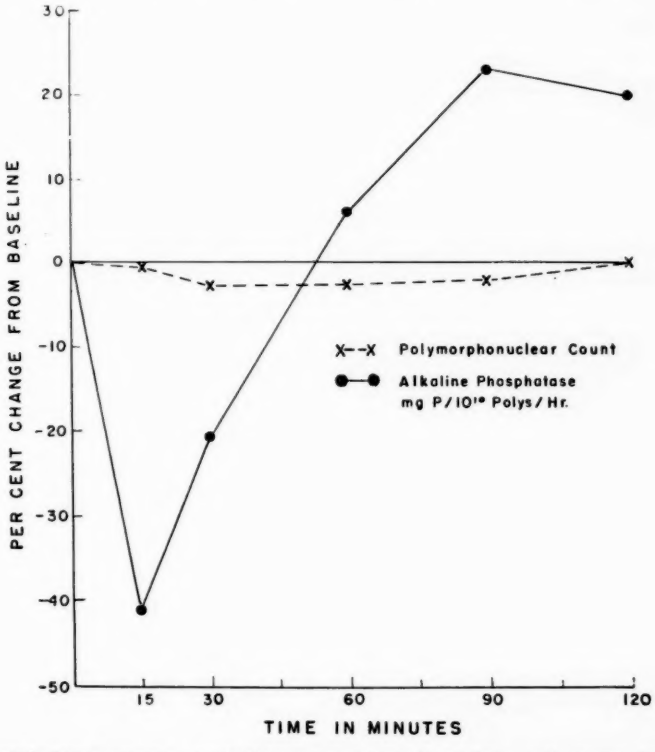

Fig. 4.—Prednisolone group: variation in polymorphonuclear cell count and alkaline phosphatase after administration of 40 μg of prednisolone intravenously.

DISCUSSION

The leukopenia that follows the administration of pyrogens has been known for a long time and is well documented. That this leukopenia may be caused by retention or sequestration of the polymorphonuclear cells in the capillaries of the lungs, liver, and spleen was already postulated by Jolly, in 1923,[10] and later, by others. Similarly, the leukocytosis seen following the administration of epinephrine is also well known. In 1923, Naegeli[11] devoted considerable space in his treatise on blood disorders to discussion of the leukocytosis observed with epinephrine, and attributed the increase in cell count to the release of cells from sequestration sites. " . . . young, immature cells were never found even with the most careful examination. It seems certain therefore that new cell formation is not the cause of the leukocytosis but rather the release of cells from the liver, spleen, lymph nodes, etc."

The changes in alkaline phosphatase and glycogen content of rabbit leukocytes after the administration of dead *B. coli* were studied by Fleck[12, 13] during the course of his investigation in leukergy. He concluded that very

soon after stimulation the leukopoietic organs delivered into the circulation peculiar energetic leukocytes rich in glycogen and enzymes (glycogenolysis). These experiments were, however, more chronic than those reported in this investigation. Blood samples were obtained hourly for the first day and then at less frequent intervals for the next 3 days. No controls were shown and the effect in the rabbit of frequent blood sampling on the white cell count and on the leukocyte glycogen and phosphatase content was not given. It would indeed be difficult to compare the results of the present study with those reported by Fleck.

The role and significance of the medullary or extramedullary reservoir sites in the fluctuations of the circulating leukocyte population have not yet been defined. Craddock and associates[14] concluded from their studies that the marrow is the chief contributor to the leukocytosis that occurs after either pyrogen administration or leukophoresis. They obtained, however, no appreciable "left shifts," but concluded from their experiments with P^{32}-tagged leukocytes that the cells came from a large marrow reserve. Bierman,[15] Andrus,[16] and others attributed to the lung a very active filtration role; it regulates the level of white blood cells. More recently, Athens[17] concluded from his studies on granulocytes labeled with radioactive diisopropyl fluorophosphate that the blood granulocyte pool consists of two compartments in rapid equilibrium with each other, namely, the circulating granulocytic pool and the marginal granulocytic pool, and further, that cells shift from the marginal pool into the circulating pool after epinephrine or exercise.

The results of the present study confirm again the change in cell count seen in the brief leukopenia and leukocytosis produced by pyrogens and epinephrine, respectively. The data also tend to support the concept that the change in cell count is due to the flow of cells into and out of sequestration sites, and further, suggest that the sequestration is not random but selective. The changes observed in the leukopenia and leukocytosis under discussion are best explained by preferential shifts of the polymorphonuclear elements more rich in alkaline phosphatase from the blood stream into sequestration sites and the return of the same or similar previously sequestered cells into the blood stream. The bone marrow plays little if any role in the phenomenon except as a site for the sequestration of cells. It would thus seem possible to determine the direction of movement of polymorphonuclear cells between marrow, blood, and the capillary sequestration sites by means of serial alkaline phosphatase assays of the polymorphonuclear leukocytes. Thus, when the cell count is increasing rapidly with a concomitant fall in the unit alkaline phosphatase, increased flow from the bone marrow of cells poorer in enzyme can be postulated. Conversely, concomitant increases of cell count and of alkaline phosphatase activity would speak for a greater flow of cells from the sequestration sites. Similar reasoning would be applicable in the acutely induced leukopenic state.

The change in alkaline phosphatase activity of the polymorphonuclear cells following the administration of steroids is more difficult to explain. No

shifts in circulating cell population were apparent in the present investigation, and preliminary studies on the direct effect of the steroids on enzyme action have given negative results. Valentine and associates[18] have shown that steroid administration raises the level of unit phosphatase activity of the circulating leukocyte. Maximum increase of enzyme activity developed gradually, and a lag period of several hours to a day preceded the start of increased activity. A definite tendency toward neutrophilic leukocytosis was noted in normal subjects who had received the steroids for 72 hours. It is interesting to note that one patient with aplastic anemia and marked granulocytopenia responded no differently to steroid administration than did the subjects with normal marrow function.

Soylemezoglu and Wells[19] noted the superficial resemblance of the leukocyte response following the administration of the bacterial pyrogens to that produced by ACTH. From their investigations on adrenalectomized dogs, they concluded that a pyrogen produces its characteristic change in white blood cell count by a mechanism other than pituitary adrenal discharge. Further, their data demonstrate that the normal dog, like the subjects in this study, shows no change in white blood cell count during the first 120 minutes after the administration of ACTH.

With the information currently available, the change in leukocyte alkaline phosphatase observed after the administration of either single or multiple injections of steroids is difficult to explain. However, the possibility that the hormone does act in part by diverting cells from capillary sequestration sites to the blood deserves further exploration.

SUMMARY

The administration of pyrogen to man produces a fall in the polymorphonuclear cell count and unit alkaline phosphatase activity. The injection of epinephrine induces a rise in both count and enzyme activity. These responses are best explained by preferential sequestration or delivery of the enzyme-rich polymorphonuclear cells.

The acute changes in alkaline phosphatase activity of the polymorphonuclear cells following steroid administration are described. The mechanism for these changes is not understood and work is being continued in this direction.

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PREVALENCE OF ABNORMAL HEMOGLOBINS IN AMERICAN INDIAN CHILDREN

Survey in the Rocky Mountain Area

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Blood samples from 338 American Indian children from Wyoming and Montana were studied for abnormal hemoglobins by paper electrophoresis. Two cases of hemoglobin D trait were discovered in one family. A further survey of their relatives revealed a total of five cases of hemoglobin D trait. Tribal records showed them to be 25/32 Shoshone and Ottawa Indian, but the hemoglobin D appeared to be transmitted through the father, who was ¾ Ottawa Indian and ¼ French Canadian. The presence of hemoglobin D is of interest since Indian ancestry has been reported in other families with hemoglobin D. The over-all low incidence of abnormal hemoglobins in these Indians of the western Rocky Mountains (primarily of the Shoshone and Arapaho tribes) confirms the previously reported low incidence of this trait in Indians of the southeastern and southwestern United States.

RECENT studies for abnormal hemoglobins in various racial groups have revealed information that is of interest not only to the physician and the geneticist, but also to the anthropologist. The American Indian represents an ethnic group of particular importance for such a survey because it may have originated in Asia, where a high incidence of abnormal hemoglobins has been found. The purpose of this communication is to present the results of hemoglobin analysis of American Indians in the northern Rocky Mountain states.

Two similar surveys of the American Indian and one study of Alaskan natives for abnormal hemoglobins have been reported recently. In 1957, Rucknagel[1] described hemoglobin electrophoretic studies in 163 blood samples from Indians of the southeastern United States and in 96 samples from Indians of the Southwest. He found three instances of sickle cell trait and no other abnormal hemoglobins. He notes that Negro ancestry could not be completely ruled out in this group of individuals. Pollitzer and associates[2] analyzed blood samples from two American Indian populations in North Carolina. These were comprised of 534 Cherokees, who showed no hemoglobin abnormalities, and 1,332 Lumbee Indians "of less certain ethnic status," of whom 1.7 per cent had hemoglobin S; an equal number had hemoglobin C. One was reported as

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having "possible hemoglobin D trait." Scott and co-workers[3] surveyed 708 Eskimos, 200 Aleuts, and 44 Indians in Alaska and found no instances of abnormal hemoglobins.

There also have been a few reports of American Indian ancestry in families with abnormal hemoglobins. Chernoff and co-workers[4, 5] found a history of Indian blood in Negroes with hemoglobin C and hemoglobin D. Thalassemia was discovered in an American Indian child in 1950,[6] and Indian ancestry was noted in the original patient in whom hemoglobin D was discovered.[7]

MATERIALS AND METHODS

Blood samples were obtained for study from 196 Indian children on the Wind River Indian Reservation in Wyoming.* Tribal records were very complete for this group and showed that they were primarily from the Shoshone and Arapaho tribes. A number of families also had white or other Indian ancestry including Ute, Navajo, Assiniboiu, Bannock, Montana Cree, Ottawa, Sioux, Crow, Pawnee, Cherokee, Pomo, and Gros Ventre. One hundred and sixteen of these children were at least ¾ Indian; thirty-eight were full-blooded Indians.

Another series, of 142 samples, was obtained from children living in the Indian settlement in Great Falls, Montana.† Information regarding the exact degree of Indian ancestry was not available for most of this group. They traced their background primarily to the Montana Cree, Blackfeet, and Chippewa tribes.

Samples of venous blood were collected in tubes containing oxalate and the red cells were separated immediately. The hemoglobin was prepared for paper electrophoresis, and whenever possible, the electrophoresis was carried out immediately; otherwise, the samples were frozen, packed in dry ice and transported to Denver.

Paper electrophoresis was performed with glass plates, in a barbital buffer at pH 8.6 with an ionic strength of 0.05, according to a modification of the method of Chernoff.[9] Fetal hemoglobin was determined on those blood samples in which paper electrophoresis suggested a significant increase in fetal hemoglobin by the alkali denaturation method of Singer and associates.[10]

RESULTS

The 196 samples from the Indians of the Wind River Reservation in Wyoming revealed two abnormal specimens. These occurred in brothers, and both blood samples contained hemoglobin A and another hemoglobin which migrated on paper at pH 8.6 at the same rate as hemoglobin S or D. Further study of samples of their blood revealed a negative sickling test. Solubility studies were done on the hemoglobin of these brothers by Goldberg's modification of the method of Itano.[11] This study confirmed the diagnosis of hemoglobin D.

The 142 samples from the Cree, Blackfeet, and Chippewa Indians of Montana revealed only hemoglobin A. A few infants in both groups showed high levels of fetal hemoglobin but these were determined to be within normal limits for their age.

*Samples were obtained as part of a health survey carried out by the Wyoming State Health Department, the Division of Indian Health, Billings, Mont., the United States Public Health Service, and the Children's Bureau of the United States Department of Health, Education, and Welfare.[8]

†Samples were obtained as part of a health survey carried out by the Cascade County Health Department with the cooperation of the Montana State Board of Health, the United States Public Health Service, and the Children's Bureau of the United States Department of Health, Education, and Welfare.

Study of the parents and five other members of the family in which hemoglobin D was discovered showed the presence of the hemoglobin D trait in the father and in two other siblings. Thus, a total of five cases of hemoglobin D trait were found in this family. Tribal records revealed them to be 25/32 Shoshone and Ottawa Indian. A more detailed analysis of their ancestry showed that the father (who had the hemoglobin D trait) was an Ottawa Indian. His father was a full-blooded Ottawa and his mother was half Ottawa and half French Canadian.

DISCUSSION

The discovery of a low incidence of abnormal types of hemoglobin in certain Indian tribes of the northern Rocky Mountain area is consistent with the previously noted low incidence of such abnormalities in Indians of the southeastern United States,[1, 2] the southwestern plains,[1] and Alaska.[3] Only two Indian children out of 338 had an abnormal hemoglobin; this was inherited from their father, who was an Ottawa, not a Plains Indian. It is of interest that the abnormality was hemoglobin D, since American Indian ancestry has been reported in four "Negro" families with hemoglobin D,[5] in the first reported case of hemoglobin D,[7] and in a member of the Lumbee tribe in North Carolina.[2]

The discovery of five cases of hemoglobin D trait in this Shoshone-Ottawa family lends support to previous suggestions that the American Indian population could be a reservoir of hemoglobin D. On the other hand, hemoglobin D has been found in both the heterozygous carrier state and in combination with other abnormal hemoglobins such as S and the thalassemia trait in almost every part of the world, whenever large population groups are surveyed. In this country hemoglobin D has been found principally in Negro families (including those with Indian ancestry),[12] but the majority of reports from other parts of the world have described its occurrence in non-Negroes. Its greatest incidence in a single ethnic group is in India where as many as 2 per cent of Sikhs have been shown to have hemoglobin D.[13, 14] It has also been reported in Algerians,[15] Turks,[16] Gujerati,[17] Filipinos,[18] Persians,[19] and several white families with European ancestry.[20-25]

However, Benzer and associates[26] have recently shown by the "fingerprinting" technique that three different samples of hemoglobin D varied slightly from each other in their peptide pattern although they resembled each other in electrophoretic mobility, resin chromatography, and solubility. These three samples (from a Sikh, a Gujerati Indian, and a Turkish Cypriot) were apparently the result of three distinct mutations. Thus, any anthropologic interpretation of the distribution of hemoglobin D must be made with caution. If the previous reports of the presence of hemoglobin D in American Negroes also reflect the same heterogeneity of this molecule, it is even less likely that the American Indian is a reservoir of hemoglobin D.

The absence of hemoglobin S and C in the Indians of the Rocky Mountain area is at variance with the finding of these hemoglobins in the Indians of the southeastern United States. This, of course, suggests that there may be less admixture with Negro ancestry in the western Indians.

SUMMARY

Hemoglobin electrophoretic analysis of 338 blood samples from American Indian children living in Wyoming and Montana revealed two specimens with hemoglobin D trait. Both of these children were in the same family and further study of their relatives uncovered a total of five cases of hemoglobin D trait. The parent with the hemoglobin D was of Ottawa Indian and French Canadian ancestry. No other abnormal hemoglobins were found. This study confirms the low incidence of abnormal hemoglobins previously noted in other American Indian tribes.

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EFFECT OF HEMOGLOBIN ON ERYTHROCYTE UREA CONCENTRATION*

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Urea has long been considered a substance freely diffusible in body fluids and has been so handled in calculation of its distribution. The urea concentration in red blood cell water was compared to urea concentration in plasma water from eighteen human subjects. The concentration of urea in red blood cell water was found consistently higher than in plasma water by a ratio of 1.19 (0.02 standard error). This concentration difference was not energy dependent. Determined urea binding by human hemoglobin was 13 per cent. It was assumed that the urea concentration difference between red blood cell and plasma water was due to the binding of urea by hemoglobin in the red blood cell.

UREA has long been considered a substance so freely diffusible as to have no osmotic effect in vivo. The osmotic effect upon the renal tubule is revealed by the production of osmotic diuresis by urea infusions.[1] The demonstration that urea excretion of man and sheep is regulated by the renal tubule and is not purely dependent upon plasma urea concentration, filtration rate, or rate of urine flow[2, 3] amplifies the lack of free diffusion of urea in the nephron.

The regulation of urea excretion by the nephron does not in itself imply absence of free diffusion in body fluids. There does exist, however, evidence that there is some limitation of the diffusion of urea in blood cells. When urea in physiologic saline is given by intravenous route to the sheep or the seal there occurs intravascular hemolysis.[3, 4] This may be so severe in the seal that death ensues.[5] This is especially interesting since 5 per cent urea in physiologic saline does not cause hemolysis of sheep red blood cells in vitro.

In an attempt to evaluate the osmotic effect of urea on red blood cells an in vitro experiment was begun, its purpose being measurement of the effect of urea on red cell sodium, potassium, and water content. The study was baffled by a difference in urea concentration in red cell and plasma water.

Since it had been reported that urea concentration in red cell water equaled urea concentration in plasma water[6–9] we assumed there was an error in our method. Various analytical modifications failed to correct this apparent error. The report by Ralls[10] that urea is not equally distributed between red cell and plasma water made us focus our attention on the red cell urea to plasma urea concentration ratio.

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It was found that the ratio of urea concentration in red cell water to urea concentration in plasma water is 1.19, in close agreement with the data of Ralls, and that urea is bound by human and bovine hemoglobins.

MATERIALS AND METHODS

Heparinized blood was obtained from fasting humans by nonocclusive venesection. A portion of the blood was centrifuged immediately and plasma obtained for the determination of water content and urea and sodium concentrations. Whole blood water content, urea concentration, and hematocrit were determined. If samples were to be incubated the plasma was replaced by means of saline solution, with sodium concentration equal to determined plasma sodium concentration, with albumin added to make the solution iso-oncotic to plasma. Urea concentration was adjusted to range from 10 to 400 mg. per cent. The samples were then incubated at 0° or 37° C. and treated as though they were fresh whole blood samples when they were removed from incubation. Some samples were incubated in a nitrogen environment or with added iodoacetate or dinitrophenol. Sterile glassware was used in all incubation procedures. Blood was drawn from three subjects both before and after meals. All analyses were done in triplicate or quadruplicate. Water content was determined by drying to a constant weight under vacuum and/or at a temperature less than 105° C. to avoid the loss of volatile fats. Water contents determined by the two methods were equal. Sodium concentrations were obtained with the use of an internal standard flame photometer. Urea concentrations were measured with the microdiffusion method of Conway,[11] with ammonia blanks obtained for each sample.

The modifications of techniques used were in the pipetting and in the analysis of urea concentration. The viscous nature of whole blood led us to compare results obtained by gravimetric method with the use of determined density of the whole blood and plasma samples, and the addition of samples to the diffusion dishes by weight. Urea determinations were checked by Nesslerization, using protein-free filtrates, and by radioisotopic techniques with tracer quantities of C^{14} urea added to the samples and incubated for 12 hours at 37° C. Modifications of techniques were run as paired experiments with the "standard" technique used. During these paired runs analyses were done in quadruplicate. Agreement was obtained with each quadruplicate sample and with the different modifications.

C^{14} was counted in the gas phase by means of a proportional counter with a micro-mil window.

Solutions of bovine albumin, bovine hemoglobin, and fresh prepared human hemoglobin in 0.9 per cent sodium chloride were prepared; each contained different concentrations of urea and were dialyzed for 12 hours at 37° C. against 0.9 per cent sodium chloride, with the same initial concentration of urea as the protein solution being dialyzed. Hydrostatic force was used to minimize change in volume of the protein solution.

RESULTS

It was consistently found that the urea concentration in red cell water was higher than that in plasma water. This concentration ratio was not affected by the subject's having eaten prior to the collection of the blood sample.

Table I presents the summary data on the eighteen human subjects. The table also contains data on the binding of urea by human and bovine hemoglobin and by bovine albumin. It is of interest that a 20 per cent albumin solution bound as much urea as a 20 per cent hemoglobin solution. Preparations of red cell stroma containing no hemoglobin were found to contain no urea by the Conway microdiffusion method.

The ratio of urea concentration in red blood cell water to that in plasma water remains the same regardless of the urea concentration in the sample.

This held true for the range of urea concentrations from 10 to 400 mg. per cent. It would thus appear that the quantity of urea bound was related to the urea concentration, indicating that the urea-binding sites on hemoglobin could not be saturated in this range urea concentration.

TABLE I. RATIO UREA CONCENTRATIONS EXPRESSED AS CONCENTRATION IN WATER

	HUMAN		BOVINE	
	R.B.C. H_2O / PLASMA H_2O	20% HGB. IN 0.9% NaCl / 0.9% NaCl	20% HGB. IN 0.9% NaCl / 0.9% NaCl	20% ALBUMIN IN 0.9% NaCl / 0.9% NaCl
Mean	1.19	1.13	1.09	1.09
Range	1.07 to 1.38	1.07 to 1.21	1.06 to 1.12	1.08 to 1.10
Number	18	3	5	2
Standard error	0.02			

Incubation of blood for 24 hours in sterile containers did not increase the red blood cell urea concentration. The ratio of urea concentration of red cell water to that of plasma water was not altered by maintenance of the sample at 0° C., by placing dinitrophenol in the medium, or by placing iodoacetate in the medium in concentrations not exceeding 0.01 M., or by maintaining the incubation in a nitrogen environment. Higher concentrations of iodoacetate were found to inhibit the urease reaction. This was so striking that 0.1 M. iodoacetate produced an 80 per cent inhibition in the urease reaction.

Van Slyke[6] stated that urea concentration in red cell water was equal to urea concentration in plasma water. He found no difference in this relationship between the arterial and renal venous blood samples.[12] When the data of Marshall,[7] Wu,[8] and Berglund[9] were recalculated from the mean water content of plasma (91 per cent) and red cells (63 per cent) as determined in our laboratory, the range of their ratios of urea concentration in red cell water to urea concentration in plasma water was 1.10 to 1.38. This agreed with our data.

DISCUSSION

It has been shown by other investigators that protein will bind urea.[13] Since the difference in urea concentration in red cell water and plasma water does not appear to be an energy-demanding process, it is assumed that this concentration difference is produced by the binding of urea by hemoglobin. Since the effects of equal concentrations of hemoglobin and albumin on the ratio of urea concentration in the protein solution to that of the dialyzing bath are the same, it must be assumed that the difference in urea concentration in red cell water and in plasma water is related to the difference in concentration of protein in the red cell and in plasma.

In 1928, Addis[14] reported that whole blood caused an error in urea determinations when performed by the urease method. The current use of a more purified urease preparation, the good recoveries of urea obtained from blood samples, and the agreement of the data obtained by Nesslerization and the

Conway microdiffusion method make us believe that the increased urea concentration in red cell water found was not due to an error induced by determination in whole blood.

The existence of the difference in urea concentration in red cell water and urea concentration in plasma water makes difficult the interpretation of data which assume equal concentration of urea in tissue water and perfusing fluids.

Our data are in agreement with the data of Ralls[10] except that we did not find a difference in the concentration ratio in the postprandial as compared to the fasting state.

SUMMARY

1. Urea concentration in red cell water is higher than urea concentration in plasma water in human blood samples.

2. This difference is presumed to be due to the binding of urea by hemoglobin and does not require energy.

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DETECTION AND ISOLATION OF INDOLEACETAMIDE FROM HUMAN URINE: RESULTS WITH SCHIZOPHRENIC AND NORMAL SUBJECTS

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Alkaline (pH 10) ether extracts obtained from human urine saturated with ammonium sulfate were found to contain a single well-defined unknown indole (Ehrlich benzaldehyde reactor) which could be measured colorimetrically with 2 per cent Ehrlich benzaldehyde reagent at 535 mμ. In a comparison of three different groups of schizophrenic and normal subjects, appreciably higher levels (optical density values) of this compound were found in extracts of the urine of schizophrenic subjects. Group A (Veterans Administration urine pools) was significant at approximately the 5 per cent level; Group B (Veterans Administration individual urine specimens), beyond the 1 per cent level; and Group C (National Institutes of Health individual urines of a "blind" study), at the 5 per cent level. Group C was of special interest because it was a "blind" ward study in which dietary factors were carefully controlled and no drugs were administered for a period of 2 months prior to test. The unknown indole has been isolated and identified as indoleacetamide. Details of the isolation procedure and identification tests used are presented. The significance of indoleacetamide in human urine is discussed.

A PREVIOUS report from this laboratory outlined a method for the differential extraction of indoles from the urine of schizophrenic and normal subjects.[1] Urine was extracted under alkaline conditions (pH 10), first with ethyl ether and then with 2-butanone. The butanone extracts were found to contain a compound tentatively identified as 6-hydroxy-skatole sulfate,[2, 3] which occurred more frequently and with greater intensity in the urine of schizophrenic patients than in that of normal subjects. The ether extracts were found to contain a single well-defined unknown indole (Ehrlich benzaldehyde reactor) which resembled tryptamine but was not the same thing, as shown by tests with paper chromatography. This unknown indole was found consistently in the urine of both schizophrenic and normal patients. However, in 6 out of 8 urine pool comparisons (4 urine specimens per pool), optical density readings taken at 535 mμ on the Beckman DK2 recording spectrophotometer gave higher values for the urine pools of schizophrenic subjects.

In view of this finding with ether extracts, it was felt desirable to: (1) examine further this apparent difference with *individual* urines from schizophrenic and normal subjects and (2) attempt to isolate and identify the unknown indole involved.

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MATERIALS AND METHODS

Procedure for Testing Ethyl Ether Extracts of the Urine of Schizophrenic and Normal Subjects.—Urine specimens were collected from the subjects, who had fasted overnight, and processed as previously described.[1] In the present instance, however, urine samples were not pooled but run individually. Each of the groups tested consisted of subjects of two types: (1) normal male members of hospital personnel and (2) male patients, 20 to 50 years of age, who had had recurrent schizophrenia over a period of years and were acutely ill at time of test. Mostly, these patients were newly admitted or *readmitted* to this hospital. Generally, there was no history of medication for at least 2 months or more prior to test, except in 3 instances in the Group B series (Table I) where two patients were off medication for 5 days and one for 3 days prior to test. This is discussed more fully under Results.

A few minor modifications in procedure were introduced to permit the processing of smaller urine volumes. Details are as follows: For each urine sample tested, a creatinine determination was first made by the alkaline picrate method and expressed as milligrams per milliliter. The entire specimen was then concentrated in vacuo (37° to 40° C) and equated to a creatinine concentration of 15 to 20 mg. per milliliter. The concentrate was then centrifuged, filtered, and the residue washed with H_2O (3 times 1/10 volume of filtrate.) The filtrate and washings were pooled, saturated with $(NH_4)_2\ SO_4$, again filtered, adjusted to pH 10 with 40 per cent NaOH, and total volume noted. The pH 10 solution was extracted 4 times with an equal volume of ethyl ether. The ether extract was then evaporated to dryness, and made up in 95 per cent ethanol to a volume equivalent to 100 mg. creatinine per milliliter.

The assay in the Beckman DK2 recording spectrophotometer was carried out as follows: 0.2 ml. of ether extract ($\simeq$ 20 mg. creatinine) were evaporated to dryness, 2 ml. of H_2O were then added, followed by 2 ml. of 2 per cent Ehrlich benzaldehyde reagent (EBR = 2 Gm. p-dimethylamino-benzaldehyde dissolved in concentrated HCl, specific gravity = 1.19). Total cuvette volume was 4 ml. ($\simeq$ 5 mg. creatinine per milliliter). Optical density readings were made at 535 mμ wavelength exactly 15 minutes after addition of the Ehrlich reagent. Results are set forth in Table I.

Procedure for Isolation of Unknown Indole in Ether Extracts.—Urine specimens were collected from a number of normal subjects who had fasted overnight, and were pooled to a total volume of approximately 3 or 4 L. A given urine pool was concentrated in vacuo 15 to 20 fold, centrifuged, filtered, and subjected to the same procedure as described in the section above, inclusive of the step in which the ether extract was evaporated to dryness. The dry ether extract was dissolved in 10 to 20 ml. of 95 per cent ethanol to the saturation point with gentle heat (40° to 45° C).

The entire extract was then applied as a band on 6 to 8 sheets of Whatman No. 1 chromatography paper (18½ in. by 22½ in.) and subjected to unidimensional descending paper chromatography overnight in isopropanol/ammonium hydroxide/H_2O in ratio of 8:1:1. Next morning, the papers were removed and dried. A vertical strip (1 cm.) was cut out from the center of each paper and sprayed with 2 per cent Ehrlich-nitrite reagent[1, 4] to determine the location of the unknown indole band on the parent sheet. The 2 per cent Ehrlich-nitrite spray consistently showed only two spots: the single unknown indole (R_f value, 0.70) as a blue spot and urea (R_f value, 0.45) as a yellow spot. Horizontal strips (5 cm. wide) were cut out from the parent sheet along a line centered at an R_f value of approximately 0.70, the location of the indole band. These strips were eluted with the isopropanol/ammonium hydroxide/H_2O solvent system used above. The eluates were pooled, evaporated to dryness, weighed (yield: 50 to 100 mg. dry material), and dissolved in 95 per cent ethanol to give roughly 5 mg. per milliliter. Solution in 95 per cent ethanol and evaporation to dryness was repeated 2 or 3 times to rid the material thoroughly of isopropanol-ammonium hydroxide.

The dry residue was emulsified by addition of 5 ml. ethyl ether and 5 ml. H_2O, and the emulsion was evaporated to a 2 ml. volume. A flocculent brown precipitate formed

which was negative to 2 per cent Ehrlich benzaldehyde reagent. This was removed by the addition of H_2O to make 4 ml., centrifuging, and washing with 1 ml. H_2O. The supernatant (approximately 5 ml., Ehrlich positive) was then extracted with 5 ml. portions of $CHCl_3$ until no Ehrlich reaction was detectable in the supernatant (6 or 7 extractions were necessary). The $CHCl_3$ extracts were pooled, evaporated to dryness, and weighed (yield 10 to 20 mg. dry material). This material showed a strong positive Ehrlich test. The dry material was redissolved in 10 ml. absolute alcohol and again evaporated to dryness to remove the $CHCl_3$. The resulting residue was then dissolved in the smallest volume of absolute ethanol effecting solution (0.2 to 0.4 ml.). Successive portions of petroleum ether were added in approximately 2 ml. amounts to a total volume of 10 to 20 ml. A white crystalline sediment (isolate), strongly Ehrlich positive, formed. Successive crops were removed by centrifugation for identification tests (see Results). The total yield from 3 or 4 liters of urine was 6 to 10 mg.

TABLE I. OPTICAL DENSITY READINGS (× 1,000) AT 535 mμ OF ALKALINE ETHER URINE EXTRACTS TREATED WITH 2 PER CENT EHRLICH BENZALDEHYDE REAGENT (NUMBERS IN PARENTHESES ARE RANK NUMBERS)

	GROUP A		GROUP B		GROUP C	
	SCHIZOPHRENIC	NORMAL	SCHIZOPHRENIC	NORMAL	SCHIZOPHRENIC	NORMAL
	370 (13.5)	260 (8)	425 (11)	262 (1)	472 (13)	298 (4)
	171 (2)	170 (1)	443 (13)	310 (4)	333 (7.5)	218 (1)
	380 (15)	282 (11)	397 (10)	328 (7)	333 (7.5)	248 (3)
	550 (16)	255 (6)	338 (9)*	318 (6)	310 (5)	328 (6)
	270 (10)	248 (5)	471 (14)	335 (8)	392 (10)	240 (2)
	370 (13.5)	258 (7)	312 (5)*	297 (3)	452 (11)	467 (12)
	290 (12)	262 (9)	428 (12)*	279 (2)	490 (14)	362 (9)
	223 (4)	205 (3)	—	—	—	—
Mean:	328	243	402	304	397	309
± Standard error	39	12	20	9	26	30
Rank No. total:	(86)	(50)	(74)	(31)	(68)	(37)
Probability level†	.05 (approx.)		< .01		= .05	

*Patients with Rank Nos. 5 and 9 were off medication for 5 days; patient with Rank No. 12 was off medication for 3 days; all other patients in this series were off medication for at least 2 months prior to test.

†Probability level refers to level of probability of difference between schizophrenic and normal optical density readings (× 1,000) as measured by Wilcoxon's method[5] of rank numbers of unpaired replicates.

RESULTS

Results With Ether Extracts of Urine From Schizophrenic and Normal Subjects.—The optical density values of ether extracts of three groups of schizophrenic patients and normal subjects are compared in Table I. These groups were as follows: *Group A* (Veterans Administration patients' pool) consisted of eight urine pools from schizophrenic patients and eight normal urine pools (4 urine specimens per pool); *Group B* (Veterans Administration individual patients) consisted of seven schizophrenic and seven normal individual urine specimens from subjects other than those used in Group A; *Group C* (National Institutes of Health individuals), consisted of seven schizophrenic and seven normal individual urine specimens selected on a "blind" basis from the wards of the Laboratory of Clinical Science, National Institutes of Mental Health, Bethesda, Maryland.

Group A was the first series studied. Although many patients of this series were newly admitted or readmitted with no recent history of medication, an appreciable number may have been off medication for a period of only 3 days prior to test.

In the studies dealing with individual urine samples, namely, Group B and Group C, an attempt was made to rule out factors such as medication, muscle mass, and dietary differences. In the Group B series, four patients were new admissions with no history of medication for at least 2 months prior to test, two patients (Rank No. 5 and 9) were off medication for 5 days, and one patient (Rank No. 12) was off medication for 3 days prior to test. It will be noted that the corresponding optical density values of the three recently medicated patients do not show a consistent deviation within the Group B series, and fall into a range of values found in some of the untreated schizophrenic patients of the Group C series. It is, therefore, doubtful whether medication affected the optical density values of these three patients. Again, in Group B, the mean excretion of urinary creatinine for the seven schizophrenic patients was 2.1 mg. per milliliter of urine and 80.7 mg. per hour for an average overnight period of 7.8 hours; for the seven normal subjects, the mean creatinine output was 2.0 mg. per milliliter of urine and 76.8 mg. per hour for an average overnight period of 7.7 hours. On the basis of milligrams of creatinine per milliliter of urine, our values compare favorably with the results of Young and associates[6] who found this mean value for eighteen schizophrenic patients to be 1.99 and for eleven normal subjects to be 2.35. On the basis of milligrams of creatinine per hour of excretion, our values compare favorably with calculations made from data of Vestergaard and Leverett[7] (1.80 Gm. creatinine per 24 hours) which come to 75 mg. per hour over a 24 hour period. Finally, in Group B, the mean (range in parentheses) age, weight, and height of the seven schizophrenic patients were 33 years (23 to 40), 170 pounds (134 to 236), and 5 feet, 8 inches (5 feet, 2 inches to 6 feet, 4 inches), respectively; for the seven normal subjects, these values were 32 years (24 to 46), 162 pounds (125 to 185), and 5 feet, 8 inches (5 feet, 4 inches to 6 feet), respectively; it would appear, therefore, that the schizophrenic patients of Group B did not differ much in muscle mass from the normal subjects of this group or of groups studied by other investigators. Since the mean age, weight, height, and urinary creatinine output of both schizophrenic and normal subjects in Group B were found to be so remarkably alike, urinary creatinine was especially suitable as a base line for evaluation of indole excretion in this series.

Results with the Group C series are of special interest, because it was run as a "blind" ward study, the identity of the test subjects being revealed only after the completion of tests. Medication was not a factor since no drugs were administered for a period of about 2 months prior to test. The normal controls of this series were volunteers who lived in the same wards under the same conditions and on approximately the same diet as the schizophrenic patients. A cursory survey revealed no correlation between optical density values and the degree of smoking, coffee drinking, or predisposition to constipation. The mean urinary creatinine excretion in Group C was as follows:

for schizophrenic patients, 2.1 mg. per milliliter of urine and 78.3 mg. per hour over an average overnight period of 10 hours; for normal subjects, 1.5 mg. per milliliter of urine and 56.6 mg. per hour over an average overnight period of 10 hours. It is noteworthy that the mean optical density values of Group B were remarkably akin to the mean optical density values of Group C with respect to both the schizophrenic patients and normal subjects.

Table I shows that: (1) mean values for schizophrenic patients were appreciably higher than mean values for normal subjects in all three groups tested; and (2) individual differences between values of schizophrenic and normal subjects were statistically significant when compared by Wilcoxon's method[5] of rank number totals for unpaired replicates. Group A results were significant at approximately the 5 per cent level; Group B, beyond the 1 per cent level; and Group C, at the 5 per cent level. The Wilcoxon method was used for statistical evaluation because of the skewed distribution of individual values. The calculations indicate a trend which favors the existence of a real difference between schizophrenic and normal values.

At this point it is important to state that the measurements set forth in Table I were obtained on ether extracts of an indole which had yet to be identified. Since observable differences were noted, it was deemed desirable to learn more about the nature of the indole in question.

Results With Identification Tests.—Isolation of the unknown indole in the ether extracts was accomplished as described in the preceding section. Preliminary paper chromatography experiments demonstrated the crystalline urine-isolate to give only one spot with 2 per cent Ehrlich-nitrite with R_f values of 0.70 in isopropanol/ammonium hydroxide/H_2O(8:1:1) and 0.85 in butanol/acetic acid/H_2O (4:1:5). Exactly the same R_f values with these solvent systems were found for crystalline reagent-grade indoleacetamide obtained from Mann Research Laboratories. Mixed samples of urine-isolate plus indoleacetamide were next tested in each of these solvent systems separately. Only one spot resulted in each case, thereby suggesting that only one compound was present. The crystalline urine-isolate was then compared with crystalline indoleacetamide by an additional series of confirmatory tests. The results are listed below.

1. Effect of other solvent systems: Both urine-isolate and indoleacetamide gave identical R_f values in other solvent systems. These were as follows: 0.66 in benzene/propionic acid/H_2O (20:15:1); 0.54 in 20 per cent KCl; 0.88 in 80 per cent phenol/NH_4OH (200:1); and 0.77 in isobutanol/NH_4OH/H_2O (9:1:1). Thus, both urine-isolate and indoleacetamide gave identical R_f values in a total of 6 solvent systems.

2. Melting point determinations: The melting point of the crystalline urine-isolate was checked against crystalline reagent-grade indoleacetamide on the Fisher-Johns melting point apparatus. Both were found to melt separately at 150° or 151° C. The mixed melting point was found to be 149° to 150° C. These values are in good comparison with the melting point of 149° to 151° C. for indoleacetamide reported by others.[8]

3. Effect of hydrolysis: Both urine-isolate and indoleacetamide were separately subjected to alkaline and acid hydrolysis. One to two milligrams of the compound under test were heated in 2N NaOH or 2N HCl for 30 minutes over a steam bath, cooled, neutralized, evaporated to dryness, and redissolved in 95 per cent ethanol. Paper chromatography of the extracts from both alkaline or acid hydrolysis showed only one spot with 2 per cent Ehrlich-nitrite reagent. This was identified as indoleacetic acid. Thus, both urine-isolate and indoleacetamide yielded the same hydrolytic end product, viz., indoleacetic acid.

4. Absorbance in ultraviolet light: Both urine-isolate and indoleacetamide at a concentration of 10 μg per milliliter in 95 per cent alchohol gave identical absorbance curves in the ultraviolet light and practically the same optical density values on the Beckman DK2 recording spectrophotometer. Thus both compounds showed a broad major peak at 280 mμ with optical density of 0.345 for urine-isolate and 0.351 for indoleacetamide; a sharp minor peak at 289 mμ with optical density of 0.295 for urine-isolate and 0.300 for indoleacetamide; and a shoulder at 274 mμ with optical density of 0.332 for urine-isolate and 0.336 for indoleacetamide. When absorbance was measured as a function of concentration at 280 mμ, identical curves were obtained for both urine-isolate and indoleacetamide. The range of linearity extended from 3-30 μg per milliliter per final cuvette volume.

5. Absorbance after reaction with Ehrlich benzaldehyde reagent: On the Beckman DK2 recording spectrophotometer, both urine-isolate and indoleacetamide gave identical absorbance peaks in the visible spectrum with 45 per cent Ehrlich benzaldehyde reagent[9]: viz., a sharp major peak at 545 mμ and a broad minor peak at 575 mμ. Absorbance-concentration curves of both compounds measured at 545 mμ with 45 per cent Ehrlich benzaldehyde reagent after 15 minutes were linear over a range of 3-30 μg per milliliter per final cuvette volume and ran very close together, i.e., to within a maximum difference of 10 per cent at the higher concentrations. It should be noted that a very slight shift in Ehrlich benzaldehyde reagent absorbance occurred with purification since the earlier ether extracts gave a major peak at 535 mμ rather than at 545 mμ. The 535 mμ peak was probably caused by the contaminating influence of urea. This, however, was not a serious factor in the comparison of the schizophrenic with the normal extracts since the 535 mμ peak was well defined and urea was present in amounts well beyond the point of critical concentration in both types of extracts.

6. Fluorescence measurements: On the Aminco-Bowman spectrophotofluorometer, both urine-isolate and indoleacetamide gave identical fluorescence spectra. An aqueous solution of either compound at 1 μg per milliliter gave an activation peak at 282 mμ and a fluorescence peak at 352 mμ. Fluorescence intensities of both compounds at this concentration read to within 5 per cent of each other. Fluorescence-concentration curves were linear over a range from 0.10 to 10 μg per milliliter per final cuvette volume.

Thus, it can be seen that by a series of tests involving paper chromatography, melting point determinations, hydrolysis, absorbance, and fluorescence

measurements, the urine-isolate was found to have the same qualitative and quantitative characteristics as indoleactetamide. It is therefore reasonable to conclude that the crystalline urine-isolate is probably identical with indoleacetamide.

DISCUSSION

To the best of the authors' knowledge, the work presented in this paper is the first detailed account of the chemical isolation of indoleacetamide from human urine extracts. Prior to this work, indoleacetamide had been detected in human urine by paper chromatography,[10, 11] but the compound was not isolated in a relatively pure state. Armstrong and co-workers[10] noted indoleacetamide in certain acid fractions of urine, but were not sure of its origin. Jepson[11] reported that indoleacetamide in human urine was probably derived chiefly from the ammonolysis of indoleacetylglucuronic acid; and, also, possibly, from indolepyruvic acid. Ammonolysis could occur readily under our conditions of extraction. Therefore, the presence of indoleacetamide in our extracts may serve as an index of the excretion of the major parent metabolite, indoleacetylglucuronic acid.

The appreciably higher levels of indoleacetamide found in urine extracts of the schizophrenic patients in this study deserve some comment, especially if indoleacetamide is regarded as a derivative of an indoleacetic acid conjugate. Conflicting claims of increased indoleacetic acid excretion in mental illness have long been reported, and were reviewed recently by Masuda and associates[12] and Sherwood.[13] Much of this controversy has centered about dietary differences, intestinal bacteria, and bowel eccentricities in the mentally ill. However, what has been overlooked is the fact that, heretofore, measurements of urinary indoleacetic acid have been few and were not sufficiently developed to facilitate distinguishment between the free and bound (conjugated) forms. Moreover, Milne and co-workers[14] have recently shown the excretion ratio of free to bound forms of urinary indoleacetic acid to be the result of a diffusion mechanism which involves increased clearance of the free form with rising urinary pH. In metabolic studies which deal with urinary indoleacetic acid assays, these workers suggest keeping test subjects in a state of mild metabolic alkalosis to rule out the pH effect. In view of these points, it is possible that past observations have in reality measured both free indoleacetic acid which occurs naturally and that which is liberated from the bound state in varying amounts due to the following factors: (1) differences in urinary pH and (2) varying degrees of hydrolysis which result from unsophisticated methods of urine collection, storage, or indoleacetic acid measurement.

Metabolic studies of the excretion of indoleacetic acid and its various forms in human urine have begun to appear in recent literature. Weissbach and associates[15] have shown indoleacetic acid to exist in the free and bound form in human urine; they suggest tissue degradation of tryptophan, as well as bacterial metabolism thereof, to be an important source of urinary indoleacetic acid. Interestingly enough, Jepson,[16] in an earlier report, found a fall in indican but no effect on the excretion of other indoles after intestinal sterilization in patients with Hartnup disease. Jepson's later report[11] implies that

urinary indoleacetic acid in the bound form may exist as a conjugate of either glucuronic acid or glutamine. The results of our present study lead us to pose the question whether an increased excretion of the glucuronide conjugate of indoleacetic acid (as measured by indoleacetamide) may occur in schizophrenia. This is of special interest in light of the renewed claim of the discovery of greater excretion levels of indoleacetic acid in schizophrenic patients by Masuda and associates[12] and Sherwood.[13] The answer to this question awaits the development of precise quantitative assays for indoleacetamide and indoleacetylglucuronic acid in human urine. Research is currently under way in this laboratory for development of such assays.

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A SIMPLE, SPECIFIC ONE-STAGE ASSAY FOR PLASMA THROMBOPLASTIN ANTECEDENT ACTIVITY

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A simple, specific one-stage assay for plasma thromboplastin antecedent (PTA) activity is described and its 90 per cent confidence limits for PTA levels above 45 per cent are given. The assay is based upon the ability of a test plasma to shorten the long partial thromboplastin time of PTA deficient substrate plasma in the presence of an optimal activating surface. Both the PTA deficient substrate plasma and the test plasma may be stored frozen. The levels for PTA in thirty-nine normal adults are usually distributed over the range between 63 and 136 per cent of a standard reference plasma (mean 97 per cent). Aluminum hydroxide gel adsorbs about 35 per cent of the PTA activity of normal citrated plasma.

THIS is a report on the standardization of a simple one-stage assay of plasma thromboplastin antecedent (PTA) based upon the kaolin clotting time technique of Margolis.[1] It measures the ability of a diluted test plasma to correct the long partial thromboplastin time of PTA deficient plasma in the presence of optimal contact surface. The need for PTA deficient substrate plasma has not proved to be a major limitation for plasma from patients with PTA deficiency is stable on storage if prepared properly.

MATERIALS

Preparation and Storage of Plasma.—Nine volumes of venous blood, taken with a Monocote E* coated needle and a siliconet coated syringe, was mixed with one volume of anticoagulant. The anticoagulant was made by mixing 2 parts of 0.1M citric acid with 3 parts of 0.1M sodium citrate. This anticoagulant minimizes pH rise due to carbon dioxide loss on storage.

The blood was centrifuged in the cold for 10 minutes at 10,000 r.p.m. in a Servall SS-1 centrifuge. Plasma to be stored as standard reference plasma or as substrate plasma was spun a second time to remove residual platelets.

The plasma was transferred with plastic pipettes to tightly capped 10 by 75 mm. polyethylene tubes. It was kept either at 4° C. for use that day or stored at −20° C. in 1 to 1.5 ml. lots. The pH readings at room temperature of 3 plasma samples stored for 1, 2, and 6 weeks were 7.55, 7.65, and 7.60, respectively. *Particular care was taken throughout to prevent contact of plasma with glass surfaces.*

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†G.E. SC-87, Dri-film.

Substrate plasma was prepared from several patients with PTA deficiency whose detailed coagulation studies have been published earlier.[2] *Standard reference plasma* was prepared at approximately 2 to 4 week intervals as needed from the same male subject. *Test plasma* was either assayed fresh or after storage for several days frozen at –20° C.

Diluting fluid for the standard reference and test plasmas was made by adding 1 part of 0.1M sodium citrate to 6 parts of 0.9 Gm. per cent sodium chloride solution. This diluting fluid, which has the same citrate concentration and ionic strength as plasma from citrated blood with a normal hematocrit, keeps these factors constant when plasma is diluted to various strengths.

Cephalin-Kaolin Reagent.—Cephalin is the name which has been used for an acetone insoluble, ether soluble mixture of brain phospholipids prepared as described earlier.[3] A 3.8 Gm. per cent stock suspension was stored frozen in small aliquots; the same aliquot was frozen and thawed repeatedly. A 40 mg. per milliliter stock kaolin suspension in physiologic saline was stored at room temperature in 25 ml. lots.

On the day of the assay, 0.1 ml. of stock cephalin was diluted in 3.4 ml. of veronal buffer.[3] One milliliter of this 1:35 dilution of cephalin was then mixed with 1 ml. of stock kaolin suspension to give a reagent with a final 1:70 dilution of cephalin in 20 mg. per milliliter kaolin suspension. This reagent was stable for several hours at room temperature. Because kaolin sediments rapidly, the reagent was mixed thoroughly just before it was pipetted into the clotting tube.

A chloroform extract of thromboplastin* was prepared according to Bell and Alton.[4] This material, diluted 1:100 in 20 mg. per milliliter kaolin suspension, was substituted for the cephalin-kaolin reagent in a few experiments.

A 35 mM. *calcium chloride* solution was made by diluting a 0.1M aqueous stock solution with distilled water.

Twelve by 75 mm. glass clotting tubes were used; they were washed in a boiling solution of an alkaline detergent (Haemasol).

METHOD

The clotting mixture consisted of: 0.1 ml. of cephalin-kaolin reagent (which supplied optimal platelet thromboplastic factorlike activity and optimal activating surface), 0.1 ml. of substrate plasma (which supplied all plasma clotting factors except PTA), 0.1 ml. of a 1:5 or 1:10 dilution of the test plasma (the only source of PTA), and 0.1 ml. of 35 mM. calcium chloride solution.

The test plasma was diluted in a polyethylene tube. Siliclad† coated pipettes were used for the dilutions and for pipetting the substrate and diluted test plasma into clotting tubes. Pipettes were never permitted to stand in either the substrate or the test plasma. These precautions were taken to ensure that the first exposure of either the substrate or the test plasma to an activating surface occurred in the clotting tube.

In the experiments reported later the first 3 reagents were added to the clotting tube in the order listed and incubated together for 3 minutes at 37° C. We have recently increased the incubation period to 4 minutes because we have observed slightly shorter clotting times with the longer incubation period (e.g., 76 seconds instead of 77 seconds with a 1:10 dilution of normal test plasma).

The prewarmed calcium chloride was then added to the clotting mixture and the tube was shaken once. Thirty seconds after addition of the calcium and at successive 5 second intervals the tube was picked up and tilted gently. The end point, which was read against a glass mirror with a strong overhead light, was taken as the first clumping of the kaolin. This was followed almost immediately by the formation of a solid clot. Tests were done in duplicate and averaged.

*Bacto thromboplastin, Difco Laboratories.
†Clay-Adams, Inc.

A dilution curve was made each day with standard reference plasma. For a complete curve dilutions of 1:5, 1:7.5, 1:10, 1:15, 1:20, and 1:40 were used. Because the dilution curves varied so little from day to day, a partial curve with dilutions of 1:5, 1:10, 1:20, and 1:40 was usually sufficient. The clotting times gave straight lines when plotted on log-log paper.

The 1:5 dilution was arbitrarily called 100 per cent. When the test plasma was diluted 1:5, its clotting time was converted directly to percentage from the reference curve; when the test plasma was diluted 1:10 the percentage read from the curve was doubled.

Because the dilution curve is a log plot, log per cent PTA was used in the statistical analysis of the data.

RESULTS

Selection of Optimal Concentrations for Cephalin-Kaolin Reagent.—Table I is a list of the clotting times obtained when the cephalin concentration was varied and the kaolin concentration was held constant at 20 mg. per milliliter. A 1:70 dilution of our 3.8 Gm. per cent stock cephalin suspension was selected as optimal.

TABLE I. CLOTTING TIMES WITH VARYING DILUTIONS OF 3.8 GM. PER CENT CEPHALIN*

CEPHALIN CONCENTRATION	CLOTTING TIME (SEC.)
1:10	107
1:30	85
1:50	80
1:70	75
1:100	75
1:150	82

*The test plasma was standard reference plasma diluted 1:10. Kaolin concentration was 20 mg. per milliliter.

TABLE II. CLOTTING TIMES WITH VARYING KAOLIN CONCENTRATIONS*

KAOLIN CONCENTRATION (MG./ML.)	CLOTTING TIME (SEC.)
0	101
2.5	77
5	77
10	76
20	74
30	74
40	84
80	108

*The test plasma was standard reference plasma diluted 1:10. Cephalin concentration was 1:70.

The clotting times shown in Table II were obtained when the kaolin concentration was varied and the cephalin concentration was held constant. A 20 mg. per milliliter kaolin suspension was selected as the standard on the basis of these data.

Substitution of Chloroform Extract of Thromboplastin as Partial Thromboplastin.—Fig. 1 is a plot of two dilution curves, one made with the cephalin-kaolin reagent and the other with a Bell and Alton choloroform extract of thromboplastin diluted 1:100 in 20 mg. per milliliter kaolin suspension. The lines are essentially parallel with the Bell and Alton preparation giving slightly shorter times. Therefore, it would appear that this preparation can replace cephalin as the partial thromboplastin for the assay.

Optimal Calcium Concentration.—The clotting times obtained when the calcium concentration was varied between 20 and 60 mM. are shown in Table III. A 35 mM. calcium chloride solution was selected as optimal.

TABLE III. CLOTTING TIMES WITH VARYING DILUTIONS OF CALCIUM*

CALCIUM CONCENTRATION (mM.)	CLOTTING TIME (SEC.)
20	95
25	79
30	75
35	75
40	75
45	75
50	79
60	80

*The test plasma was standard reference plasma diluted 1:10.

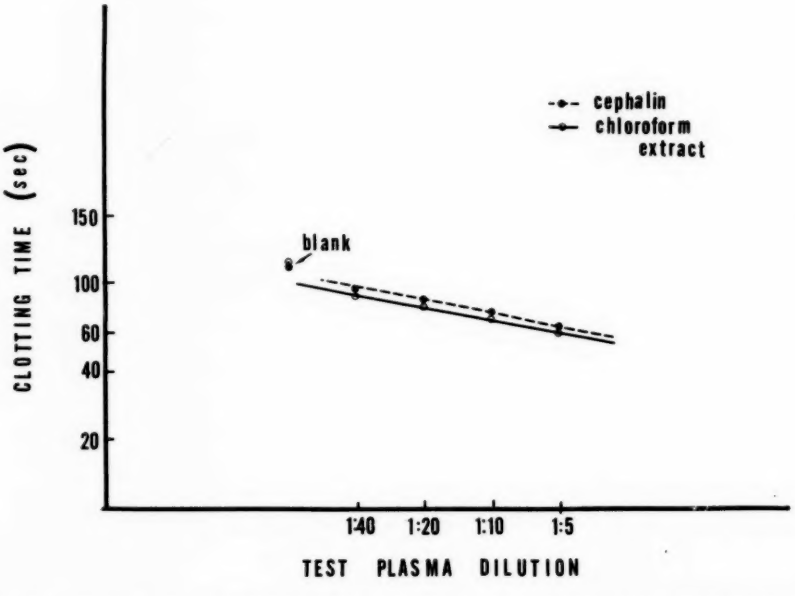

Fig. 1.—Dilution curves obtained with cephalin and with chloroform extract of thromboplastin as the partial thromboplastins.

Stability of Substrate Plasma.—Two sets of clotting times of dilutions of standard reference plasma are listed in Table IV. One was obtained with fresh substrate plasma and the other with the same substrate plasma after it had been stored frozen at –20° C. for 19 days. The two sets of clotting times are almost identical.

Different lots of substrate plasma have remained stable on storage for periods of up to 3 months. We believe that the requirements for storage stability are (1) removal of platelets, (2) prevention of pH rise, and (3) prevention of contact with an activating surface.

Fig. 2 is a plot of two dilution curves made with substrate plasma prepared from different patients with PTA deficiency. We have used substrate plasma

from a number of PTA deficient subjects and have obtained dilution curves which may vary slightly in the absolute values for the clotting times but which do not vary in slope.

TABLE IV. CLOTTING TIMES USING FRESH AND STORED SUBSTRATE PLASMA

DILUTION OF STANDARD PLASMA	CLOTTING TIME (SEC.)	
	FRESH SUBSTRATE PLASMA	STORED SUBSTRATE PLASMA (19 DAYS)
1:5	66	66
1:7.5	—	71
1:10	77	77
1:15	—	82
1:20	90	88
1:40	98	98
Diluting fluid	127	123

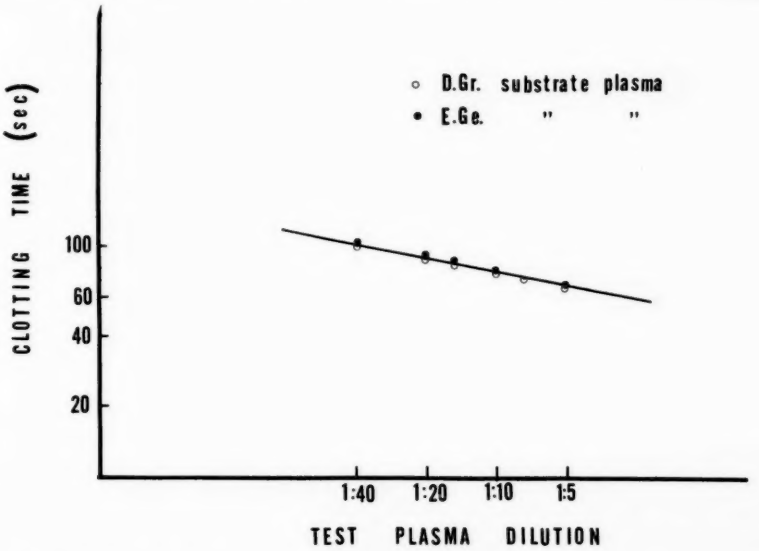

Fig. 2.—Dilution curves obtained with substrate plasma from two unrelated patients with PTA deficiency.

TABLE V. CLOTTING TIMES OF DILUTIONS OF 3 LOTS OF STANDARD REFERENCE PLASMA

DATE PLASMA DRAWN	DAYS STORED	CLOTTING TIME (SEC.) AT DILUTIONS				
		1:5	1:10	1:20	1:40	BLANK
April 26	35	65	71	85	95	116
	36	64	76	87	96	118
	37	64	75	85	95	120
	38	65	75	85	96	127
	41	64	77	87	97	120
May 23	fresh	66	77	90	98	127
	1	67	79	92	101	128
	3	67	77	91	100	122
	4	68	77	87	102	129
June 7	1	66	77	88	98	123
	3	63	75	87	98	117
	7	65	74	84	95	118
	10	67	77	88	99	137

Stability of Standard Reference Plasma.—Table V is a summary of the clotting times of different lots of standard reference plasma which were assayed repeatedly using the same lot of stored substrate plasma. Different lots of standard reference plasma behaved alike. No difference was found in the clotting times of fresh and stored standard reference plasma.

Two series of paired samples were assayed to obtain further data on the storage stability of frozen test plasma. One series, which is shown in Table VI,

TABLE VI. PTA ACTIVITY OF SAME PLASMA SAMPLE BEFORE AND AFTER STORAGE AT −20° C. FOR FROM 5 TO 10 DAYS

	PTA ACTIVITY			
	BEFORE STORAGE		AFTER STORAGE	
PAIR NO.	%	LOG %	%	LOG %
1	46	1.66	50	1.70
2	47	1.67	47	1.67
3	50	1.70	60	1.78
4	108	2.03	94	1.97
5	90	1.95	94	1.97
6	54	1.73	57	1.76
7	53	1.72	45	1.65
8	53	1.72	47	1.67
9	124	2.09	102	2.01
10	82	1.91	70	1.85
11	78	1.89	98	1.99
12	106	2.03	78	1.89
13	82	1.91	86	1.93
14	85	1.93	96	1.98
15	120	2.08	110	2.04
16	104	2.02	100	2.00
17	128	2.11	88	1.94
18	104	2.02	98	1.99
19	102	2.01	88	1.94
Mean	80		77	

TABLE VII. PTA ACTIVITY OF TWO FRESH PLASMA SAMPLES FROM SAME INDIVIDUALS ON DIFFERENT DAYS

	PTA ACTIVITY			
	SAMPLE 1.		SAMPLE 2.	
PAIR NO.	%	LOG %	%	LOG %
1	125	2.10	100	2.00
2	96	1.98	86	1.93
3	120	2.08	120	2.08
4	100	2.00	104	2.02
5	88	1.94	102	2.01
6	120	2.08	104	2.02
7	110	2.04	100	2.00
8	108	2.03	104	2.02
9	116	2.06	128	2.11
10	80	1.90	100	2.00
11	132	2.12	126	2.10
12	110	2.04	96	1.98
13	128	2.11	136	2.13
14	85	1.93	96	1.98
15	88	1.94	74	1.87
16	47	1.62	52	1.72
17	84	1.92	100	2.00
18	75	1.88	61	1.79
19	86	1.93	86	1.93
Mean	96		96	

consisted of the values obtained when the same plasma sample was assayed before and after storage at –20° C. for from 5 to 8 days. The second series, which is shown in Table VII, consisted of the values obtained when a fresh sample from the same subject was assayed on two different days. The methods of variance analysis[5] were used to compare the error of the two series. As shown in Table VIII, the two variances are of the same order of magnitude. Storage did not produce differences significantly greater than those encountered when fresh samples were assayed on different days ($p > 0.1$).

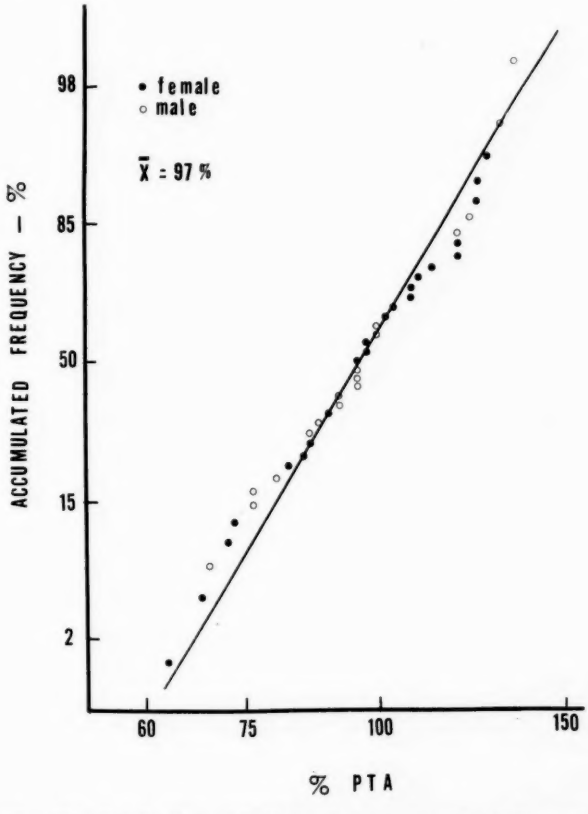

Fig. 3.—The distribution of PTA levels in a normal adult population.

PTA Levels in Fresh and Frozen Plasma of Normal Subjects.—The range of PTA activity found in thirty-nine normal adults varied between 63 and 136 per cent of our standard reference plasma (mean 97 per cent). The individual values expressed as log per cent PTA and identified by sex of the subject, have been plotted on probability paper against accumulated frequency expressed in per cent in Fig. 3. The reasonable linearity of the plot is consistent with sampling from a homogenous population. The wide range indicates that many factors must influence the PTA level of a normal subject. Our data do not identify these factors but do exclude the influence of sex.

The combined data of these two series were used to calculate the accuracy with which a single determination on either a fresh or a frozen test plasma sample estimates the true level found by making repeated single determinations on fresh samples over an indefinite number of days. This is shown in Fig. 4, which gives the 90 per cent confidence limits for PTA levels between 40 and 130 per cent of our standard reference plasma. The meaning of these confidence limits may be made more explicit with an example. If a single determination of PTA is 70 per cent, we may have 90 per cent confidence that the true level found from an indefinite number of tests on different days would have an average value that fell between 59 and 84 per cent PTA.

TABLE VIII. SUMMARY OF VARIANCE OF DATA IN TABLES VI AND VII

VARIATION	SUM SQUARES	DEGREES FREEDOM	VARIANCE
Between same sample fresh and frozen	0.0995	38	0.0026
Between two fresh samples	0.0724	38	0.0020

TABLE IX. PTA LEVELS IN PATIENTS WITH VARIOUS FIRST STAGE PLASMA CLOTTING FACTOR DEFICIENCIES

DISORDER	PTA (% OF STANDARD REFERENCE PLASMA)
Normal range (39 subjects)	63-136
Antihemophilic globulin deficiency	
D. V.	90
L. B.	66
G. T.	85
Plasma thromboplastic component deficiency	
J. G.	72
T. H.	92
D. S.	86
Hageman factor deficiency	
N. E.	71
PTA deficiency	
M. M.	6
M. T.	12
E. F.	10
L. J.	10
E. G.	7
R. K.	3
S. S.	10
R. J. K.	3

Adsorption of PTA From Citrated Plasma by Aluminum Hydroxide Gel.— It is well known that various prothrombin adsorbents partially adsorb PTA from plasma. Our routine adsorption technique is to add 0.1 ml. of a 1:4 dilution of an aluminum hydroxide gel* to 1 ml. of citrated plasma, to incubate for 3 minutes at 37° C., and then to remove the aluminum hydroxide by centrifugation. When 9 normal intact plasma samples were treated in this fashion,

*Cutter Laboratories, Berkeley, Calif.

using silicone technique throughout, a mean of 35 per cent of the original PTA activity of the plasma was removed. The loss in individual samples varied between 12.5 and 43 per cent.

PTA Levels in Pathologic Plasmas.—Examples of the PTA levels found in patients with a variety of first stage plasma clotting factor deficiencies are given in Table IX. Values within the normal range were found in every disorder except PTA deficiency. The patients with PTA deficiency had values between 3 and 12 per cent of normal.

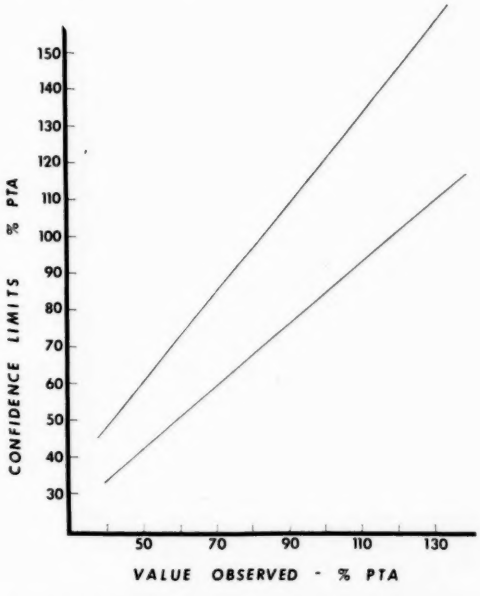

Fig. 4.—Ninety per cent confidence limits for PTA levels above 45 per cent of our standard reference plasma.

DISCUSSION

The first step in the generation of blood thromboplastic activity in vitro is believed to be an "activation" of Hageman factor by contact with a foreign surface such as glass or kaolin.[6] Activated Hageman factor then reacts with PTA to form a new activity which has been called activation product.[7] Activation product may form in the absence of calcium ions[7]; activation product triggers the subsequent reactions leading to blood thromboplastin generation but only if calcium ions are present.

PTA deficient plasma which has been incubated at 37° C. for several minutes in the presence of an optimal activating surface has a long partial thromboplastin time because activation product does not form. When a diluted test plasma is added to the incubation mixture, the partial thromboplastin time shortens; the PTA in the test plasma permits activation product to form during the incubation period. If the other reagents in the clotting mixture supply all other factors in optimal amounts, the degree of shortening will be a measure of the PTA content of the test plasma.

This is the theoretic basis for the assay; the data presented provide evidence that it is specific and reproducible. It is reliable for quantitative studies of PTA levels above about 15 per cent of normal. However, it is not suitable in its present form for measuring trace amounts of PTA accurately, as, for example, in following the disappearance rate of transfused PTA in a patient with PTA deficiency. In this situation the dilution of the test plasma reduces its PTA activity to a level approaching that present in the substrate plasma. Preliminary experiments suggest that this difficulty can be overcome by (1) using the test plasma at full strength and (2) making the dilutions of the standard reference plasma for the dilution curve in *substrate plasma* rather than in diluting fluid.

SUMMARY

A simple, one-stage assay for plasma thromboplastin antecedent (PTA) activity has been constructed. It is based upon the ability of a test plasma to shorten the long partial thromboplastin time of PTA deficient substrate plasma in the presence of an optimal activating surface. Both the substrate plasma and the test plasma may be stored frozen. Either cephalin or the Bell and Alton chloroform extract of thromboplastin may serve as the partial thromboplastin. A normal adult population was found to have PTA levels which varied between 63 and 136 per cent of a standard reference plasma.

We wish to thank Dr. Frederick J. Moore, Professor of Public Health, University of Southern California School of Medicine, for his advice on the statistical analysis of our data. Dr. Robert Proctor helped in the collection of the normal blood samples.

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INTRACOMMUNITY ADMIXTURE OF HUMAN ASSOCIATED STAPHYLOCOCCI

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Staphylococci bred in environments high in antibacterial agent content are usually resistant to the agents characteristic of the environment of origin; staphylococci from areas low in content of antibacterial agents are usually susceptible to antibacterial agents. Although the bulk of human associated staphylococci exist in environments low in antibacterial agent content, there has been admixture with staphylococci from high antibacterial agent content environments for about fifteen years. The impact of such exchange in Salt Lake City was assayed by comparison of 232 tellurite-positive staphylococcal isolates, epidemiologically representative of this community, with regard to hemolysin, kinase and protease activity, antiserum reactivity, susceptibility to fifteen antibacterial agents, and bacteriophage type.

Fifty-nine nasal colonizing and fifty-five lesion isolates from individuals in a high antibacterial agent–usage area (hospital) significantly more often produced two hemolysins, resisted bactericidal action(s) of penicillin, and resisted inhibition by dihydrostreptomycin, chloramphenicol, tetracycline, erythromycin, and sulfadiazine than did sixty-eight nasal colonizing and fifty lesion isolates from individuals in low antibacterial agent usage environments. While over-all phage susceptibility was similar, lysis by Phages 80 or 81, singly or in combination, occurred significantly more frequently with pathogens from high antibacterial agent usage areas.

More than a decade of admixture of human associated staphylococci from differing antibacterial agent environments has not resulted in the emergence of staphylococci of uniform characteristics in the Salt Lake City community.

RESISTANCE to antibacterial agents has definite survival value for bacteria bred in an environment high in content of antibacterial agents. Hospitals particularly,[1, 2] and certain pharmaceutical plants, as well as some laboratories,[3] provide environments common to both humans and staphylococci which are characterized by high concentrations of various antibacterial agents.

On the other hand, resistance to antibacterial agents has little survival value for bacteria bred in an environment low in content of antibacterial agents. Staphylococci of extrahospital origin should be more often susceptible to antibacterial agents and, indeed, such findings have been reported.[4, 5] The bulk of

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the world's human associated staphylococci exist in environments low in content of antibacterial agents. That is, the human and staphylococcal populations of hospitals, certain pharmaceutical plants, and some laboratories are quite small in terms of the over-all demography of both of these life forms.

Admixture of human associated staphylococci from high and low antibacterial agent environments is continuous and has been going on ever since foci high in antibacterial agent content were established about 15 years ago. The lay press, with eye-catching reference to a "golden horde"[6] of antibacterial agent–resistant staphylococci, the "golden villian"[7] bacteria, has called attention to the possibility that in time only antibacterial agent–resistant staphylococci will associate with human beings.

Study of the metabolic accomplishments,[8] bacteriophage susceptibility,[9] and virulence of staphylococci[10] has established *Homo sapiens* as the major reservoir of staphylococci pathogenic for human beings. It follows that the actual ecologic impact of exchange of staphylococci which are colonizing and parasitizing human inhabitants of environments differing in content of antibacterial agents can be assayed only by careful examination of a minimum of four groups of staphylococci:

1. *High* antibacterial agent area nonpathogens.
2. *High* antibacterial agent area pathogens.
3. *Low* antibacterial agent area nonpathogens.
4. *Low* antibacterial agent area pathogens.

These four groups constitute an epidemiologic minimum suitable for comparison only if all of the staphylococci studied have been obtained from the human inhabitants of a given community during a particular, brief time span.

There are studies which compare staphylococci of intrahospital origin with those from outside the hospital environment,[2, 4, 11] but these studies have not been based on a community sampling sufficiently broad to permit epidemiologically valid assay of staphylococcal interchange within the community concerned. Moreover, comparison of intra- and extra-hospital isolates has often been carried out with regard only to antibiotic susceptibility[11] or on the basis of antibiotic and bacteriophage susceptibility.[2, 4] Other reports are restricted to comparisons of staphylococci in terms of metabolic attributes; e.g., hemolysins[12, 13] or immunologic peculiarities.[14]

Nonpathogenic and pathogenic staphylococci isolated from humans of environments differing in content of antibacterial agents have not been examined by simultaneous observation of multiple parameters. Such detailed characterization is desirable because it permits detailed comparison. With detailed comparison, the probability is enhanced for perception of whether or not significant differences mark the staphylococci of a community. It is the presence or absence of significant differences among four groups of staphylococci, obtained in a given community and constituted as described, which will give clue to the extent and effect of staphylococcal admixture within that community.

Since staphylococci are nearly always present on or in human beings, culture of a lesion clinically consistent with staphylococcal sepsis may not always

yield the cocci which are actually cause for the lesion under study, e.g., culture of sinus tract exudate may yield skin resident staphylococci but not the staphylococci of an underlying abscess. The recognition of those colonizing staphylococci commensal at the time of culture but potentially pathogenic is even more difficult. Plasma coagulation is an attribute, demonstrable in the laboratory, of nearly all pathogenic and potentially pathogenic staphylococci. Tellurite reduction[15] is of identical significance and is a test which is both easier and less expensive to carry out than is the tube coagulase test.

Two hundred thirty-two isolates of tellurite-positive staphylococci were obtained in Salt Lake City during a 9-month period (June, 1958, to April, 1959) from the following:

1. Normal medical personnel in the hospital (house staff and nurses)—59 nose isolates, i.e., *high* antibacterial agent–usage area nonpathogens.

2. Patients—55 isolates from lesions acquired after hospitalization, i.e., *high* antibacterial agent–usage area pathogens.

3. Normal high school seniors—68 nose isolates, i.e., *low* antibacterial agent–usage area nonpathogens.

4. Patients—60 isolates from lesions acquired outside the hospital in the absence of any antibacterial chemotherapy, i.e., *low* antibacterial agent–usage area pathogens.

Each isolate was examined for: α,- β,- Δ-hemolysin, kinase and protease activity, antiserum reactivity, antibacterial agent and bacteriophage susceptibility.

MATERIALS AND METHODS

Staphylococcal Isolates.—

Nonpathogens: Sterile cotton-wool swabs were moistened with sterile 0.9 per cent saline before insertion into the anterior nose. The swab was rotated against the septum and the ala nasi before withdrawal, and then deposited in a culture tube containing 1.0 ml. of sterile tryptic digest of casein-papaic digest of soybean broth. After remaining 20 to 24 hours at room temperature, the swab was used to inoculate ¼ the area of a tellurite glycine agar plate. Following 18 hours' incubation at 37° C. in a moist-air candlejar, the plates were inspected for jet black colonies. Growth from at least 5 such colonies was picked off with a straight bacteriologic wire for inoculation of 10 ml. tryptic digest of casein-papaic digest of soybean broth in 15 by 125 mm. screw-capped culture tubes. Overnight incubation at 37° C. was followed by number labeling and parafilm sealing of the tubes preparatory to freezing and storage at –22° C. The frequency of isolation of nasal colonizing staphylococci by this method was not unusual; it occurred in 50 per cent of normal medical personnel and 67 per cent of normal high school seniors. Fifty-nine of the 61 nonpathogenic isolates obtained from individuals in a *high* antibacterial agent–usage area were examined (2 were lost). Of the 123 *low* antibacterial agent–usage area nonpathogenic isolates, 68 were taken for examination by random removal of tubes from this group as they lay in a basket in the freezer chest.

Pathogens: Tellurite-positive staphylococci were obtained from patient specimens submitted to the clinical microbiology laboratory. By examination of the clinical chart of each patient from whom a specimen yielding tellurite-positive staphylococci was obtained, decision was made as to whether or not that staphylococcal isolate was a cause of disease. Those isolates, which on clinical grounds were pathogens, were further classified as to origin from lesions acquired by patients at the following times: (1) after hospitalization (55 *high*

antibacterial agent–usage area isolates); or (2) outside the hospital (most of these isolates were cultured from outpatients); an isolate was accepted in this group, if, in addition, it was established that no antibacterial agent had been given to the patient before the culture yielding staphylococci was taken (50 *low* antibacterial agent–usage area pathogens). In Table I are listed the specimens from which these staphylococci were isolated. All 105 pathogenic staphylococcal isolates were subcultured in broth and stored at −22° C., as described above.

TABLE I. LESIONS YIELDING PATHOGENIC TELLURITE-POSITIVE STAPHYLOCOCCI

SPECIMEN	NUMBER OF ISOLATES FROM PATIENTS WITH LESIONS ACQUIRED	
	AFTER HOSPITALIZATION (HIGH ANTIBACTERIAL AGENT–USAGE AREA PATHOGENS)	OUTSIDE THE HOSPITAL WITH NO ANTIBACTERIAL CHEMOTHERAPY BEFORE CULTURE (LOW ANTIBACTERIAL AGENT–USAGE AREA PATHOGENS)
Blood*	7	5
Urine	3	3
Sputum	2	2
Pus.—		
Wounds	13	4
Middle ear	5	12
Furuncles and abscesses	11	11
Pleural effusion	1	0
Osteomyelitis	1	1
Ulcer	4	0
Other lesions	8	12
Total	55	50

*A blood culture isolate was accepted as a pathogen if it was recovered from one of a series of positive cultures taken from the same patient, and if the clinical course in this patient was consistent with staphyloccocal sepsis.

Selective media work their effects through differences in the susceptibility of various kinds of bacteria to inhibitors, the toxic agents of selection incorporated in these culture media. It is possible that growth on tellurite glycine agar may have wrought permanent change in the staphylococci under study. Although every one of the 232 staphylococcal isolates examined was taken from growth on tellurite glycine agar, this possibility was investigated. Six swab cultures were planted on blood agar; from each plate, growth from 5 colonies was taken to seed a tube of tryptic digest of casein-papaic digest of soybean broth—labeled "before tellurite." After overnight growth, prior to freezing for storage, tellurite glycine–agar was inoculated from this broth culture; 18 hours later, jet black colonies were picked to inoculate storage broth cultures—labeled "after tellurite," as described previously. The same parameters were observed with these paired "before" and "after" tellurite isolates as were examined for the 232 isolates with which this report is concerned. The reactions were virtually identical. With the single concentration antibacterial agent susceptibility tests employed (see below), the 3 observed disagreements in susceptibility (of 90 tests) are not significant. One nasal isolate was only partially lysed (fewer than 50 placques) by Phage 42b and so was nontypable after tellurite, although before tellurite, Phage 42b effected confluent lysis. Exposure of staphylococci to the selective inhibitors present in tellurite glycine agar (potassium tellurite, glycine, lithium chloride) does not effect changes in the properties under examination in this study.

Hemolysins.—α-, β-, Δ-Hemolysin activities were distinguished with the use of the Petri plate-antiserum strip method of Elek and Levy.[12]

Kinase and Protease.—By means of the plasma plate–soybean trypsin inhibitor paper strip method of Hinton and Orr[14] staphylokinase and staphyloprotease activities were simultaneously detected and differentiated.

Antiserum Reactivity.—Using commercial "polyvalent" rabbit antistaphylococcal antiserum,* nutrient agar-antiserum strip cultures were prepared as described by Hinton and Orr.[14] The number of lines of interaction between diffusible products which originated from the test staphylococci and the antiserum was recorded.

Antibacterial Agent Susceptibility.—Susceptibility to 15 antibacterial agents was tested.† Selected on the basis of blood concentrations attainable in patients under clinically practicable conditions, one test concentration was used for each agent except penicillin (penicillin was tested at 2 concentrations—see Table II). Pharmacy stocks for 1959 (Table II) of those agents available in Salt Lake County General Hospital, the principle *high* antibacterial agent environment of this study, give indication of the kind and intensity of antibacterial agent exposure which this hospital provided.

TABLE II. ANTIBACTERIAL AGENTS AND TEST CONCENTRATIONS APPLIED IN CARRYING OUT TUBE SUSCEPTIBILITY TESTING IN BROTH*

AGENT	TEST CONCENTRATION IN μG/ML. (UNITS/ML. FOR BACITRACIN AND PENICILLIN)	1959 PHARMACY STOCK, SALT LAKE COUNTY GENERAL HOSPITAL
Bacitracin	1	None
Chloramphenicol	10	5,600 Gm.
Cycloserine	25	None
Dihydrostreptomycin	25	3,260 Gm. (streptomycin and dihydrostreptomycin)
Erythromycin	10	1,776 Gm.
Kanamycin	15	None
Neomycin	10	6,100 Gm. (as tablets and topical solution)
Nitrofurantoin	10	1,200 Gm.
Novobiocin	10	187 Gm.
Oleandomycin	5	None
Penicillin	50	2,440 Gm. (3,897, 500, 000 units)
	200	
Ristocetin	5	None
Sulfadiazine	150	1,025 Gm.
Tetracycline	5	4,995 Gm. (tetracycline and congeners)
Vancomycin	5	None

*The amount of each agent stocked in the pharmacy of the Salt Lake County General Hospital during 1959 is listed, and serves as an indicator of the extent of usage of these agents.

Stock solutions of each agent (except sulfadiazine) were prepared in tryptic digest of casein-papaic digest of soybean broth in a concentration 10 times greater than the final test concentration. A large number of sterile 13 by 100 mm. screw-capped tubes were then made ready at one time by delivering 0.1 ml. of a given agent per tube, labeling, and storing at −22° C. For testing (except with sulfadiazine), each isolate was grown overnight in tryptic digest of casein-papaic digest of soybean broth subculture from the frozen storage tube. According to a direct bacterial count in the Petroff-Hausser chamber, a broth dilution of the subculture was prepared such that each inoculum of 0.9 ml. contained 10,000 staphylococci.

For assay of susceptibility to sulfadiazine, the preparation of sodium sulfadiazine in appropriate concentration, the subculture of staphylococci from frozen stock, and the dilution

*Connaught Medical Research Laboratories, Toronto, Ontario.

†Penicillin, dihydrostreptomycin, chloramphenicol, tetracycline, and oleandomycin were supplied by Charles Pfizer & Company, Inc., Brooklyn, N. Y.; erythromycin, cycloserine, and vancomycin by Eli Lilly & Company, Indianapolis, Ind.; kanamycin by Bristol Laboratories, New York, N. Y.; nitrofurantoin by Eaton Laboratories, Inc., Norwich, N. Y.; novobiocin by Merck Sharp and Dohme (Division of Merck & Company, Inc.), Philadelphia, Pa.; ristocetin by Abbott Laboratories, Inc., North Chicago, Ill. Neomycin and sulfadiazine were purchased from commercial suppliers.

of subculture for inoculation of the actual sulfadiazine susceptibility test were all carried out in a special semisynthetic broth medium containing no p-aminobenzoic acid.*

Incubation at 37° C. was continued for 24 hours. Tubes with visible growth were accepted as indicative of resistance on the part of the test staphylococcus to the antibacterial agents in these tubes. From each tube without visible growth, a 3mm. in diameter loopful of the thoroughly mixed contents was inoculated on an eighth sector of a nutrient agar plate. After 24 hours at 37° C. in a moist-air candle-jar, the plates were inspected: growth was taken to indicate bacteriostasis; absence of growth was equated with bactericidal action. The validity of this sampling procedure was established as follows. With 5 of the isolates, there were 80 tubes in which there was no growth by visual inspection. Loop subculture was carried out as described above and then these 80 tubes were centrifuged at 4° C. for 1 hour (RCF at tube tip 1,300 g). Following centrifugation, all but about 0.1 ml. of the supernatant was removed by careful capillary pipette aspiration. The culture area of each tube was then washed 3 times with a single 1.0 ml. aliquot of tryptic digest of casein-papaic digest of soybean broth. This broth was then transferred to a sterile Petri dish and 15 ml. melted (56° C.) tryptic digest of casein-papaic digest of soybean agar was added and swirled to mix. The gelled cultures were incubated at 37° C. for 24 hours. There was complete agreement between loop subculture and centrifuged tube–agar plate subculture—53 tubes yielded growth; 27 tubes gave no growth.

Bacteriophage lysis: Each staphylococcal isolate was exposed to 25 bacteriophages† (29, 52, 52a, 79, 80, 3a, 3c, 55, 6, 7, 42e, 47, 53, 54, 70, 73, 75, 77, 42b, 44a, 47c, 42d, 81) according to a modification[17] of the method of Blair and Carr.[18] The conventional requirement of 50 or more placques at the routine test dilution of phage for an acceptable typing reaction was observed. In keeping with the position of the Laboratory Section, Committee on Staphylococcal Phage Typing,[19] that report of bacteriophage groups is of no epidemiologic value, the phage patterns observed were recorded without attempt at grouping (Table V).

RESULTS

An over-all summary of the information amassed concerning 232 tellurite-positive staphylococcal isolates obtained from humans of *high* and *low* antibacterial agent environments in Salt Lake City, nonpathogens and pathogens, in 1958 to 1959, is presented in Table III. The 4 groups of staphylococcal isolates were generally similar in enzymatic activity, antiserum reactivity, antibacterial agent, and bacteriophage susceptibility. However, with closer examination of the data, differences among these 4 groups of staphylococci are revealed. Only differences which by the chi-square test yielded $p \leq 0.02$ are referred to as significant.

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*Acid hydrolysate of casein (Difco Casamino Acids) 20.0 Gm.
Sodium chloride 5.0 Gm.
Dipotassium phosphate 2.5 Gm.
Glucose 2.5 Gm.
Phenolsulfonphthalein 0.0005 Gm.
Distilled water *q.s. ad* 1,000.0 ml.

The pH was set at 7.2 and the solution sterilized in the steam autoclave at 121° C.; when cooled, the following were added:

(a) Amino acids (in N HCL), 2.0 ml. per liter medium:
l-cystine, 1.5 per cent
l-tyrosine, 2.0 per cent
l-tryptophane, 0.2 per cent

(b) Minerals ($MgSO_4$, $FeSO_4$, $ZnSO_4$, $MnSO_4$ solution), prepared as described elsewhere,[16] 1.0 ml. per liter of medium.

(c) Following addition of (a) and (b), 5 N sodium hydroxide was used to restore the pH to the range 7.2 to 7.4, according to the phenol red internal indicator. Accessory growth substances were then added, using 50 ml. per liter medium of a filter sterilized mixture (biotin, nicotinic acid, pyridoxine, riboflavin, thiamin, calcium pantothenate, adenine sulfate, uracil, choline chloride, and asparagine) prepared as described elsewhere.[16]

†Bacteriophages and host staphylococci for propagation were supplied by Dr. John E. Blair, Hospital for Joint Diseases, New York, N. Y.

Enzymes.—Hemolytic activity is viewed according to kind of hemolysin in Fig. 1, and by numbers of hemolysins per isolate in Fig. 2. Staphylococci isolated from *high* antibacterial agent environments elaborated 2 hemolysins significantly more often than staphylococci isolated from *low* antibacterial agent

TABLE III. OVER-ALL SUMMARY OF DATA REGARDING 232 TELLURITE-POSITIVE STAPHYLOCOCCAL ISOLATES, NONPATHOGENS AND PATHOGENS, OBTAINED IN 1958 AND 1959 FROM HUMANS IN HIGH AND LOW ANTIBACTERIAL AGENT–USAGE AREAS IN SALT LAKE CITY, UTAH

KIND OF STAPHYLOCOCCUS AND ANTIBACTERIAL AGENT ENVIRONMENT OF ISOLATION	NUMBER OF ISOLATES	AVERAGE NUMBER, PER ISOLATE OF			PER CENT PHAGE TYPABLE
		ENZYMES*	"TOXINS"†	EFFECTIVE‡ ANTIBACTERIAL AGENTS	
Nonpathogens:					
High	59	2.6	2.3	10.2	40.6
Low	68	2.6	3.0	11.4	47.1
Pathogens:					
High	55	2.6	2.2	9.3	49.0
Low	50	2.7	1.6	9.9	46.0

*Enzymes refers to α- β-, and Δ-hemolysins, staphylokinase, staphyloprotease.

†"Toxins" corresponds to visible lines of interaction (precipitation) in agar gel of diffusible products of staphylococci with commerical, rabbit, polyvalent, antistaphylococcal antiserum.

‡Effective implies inhibition of growth, whether due to bacteriostatic or bactericidal action.

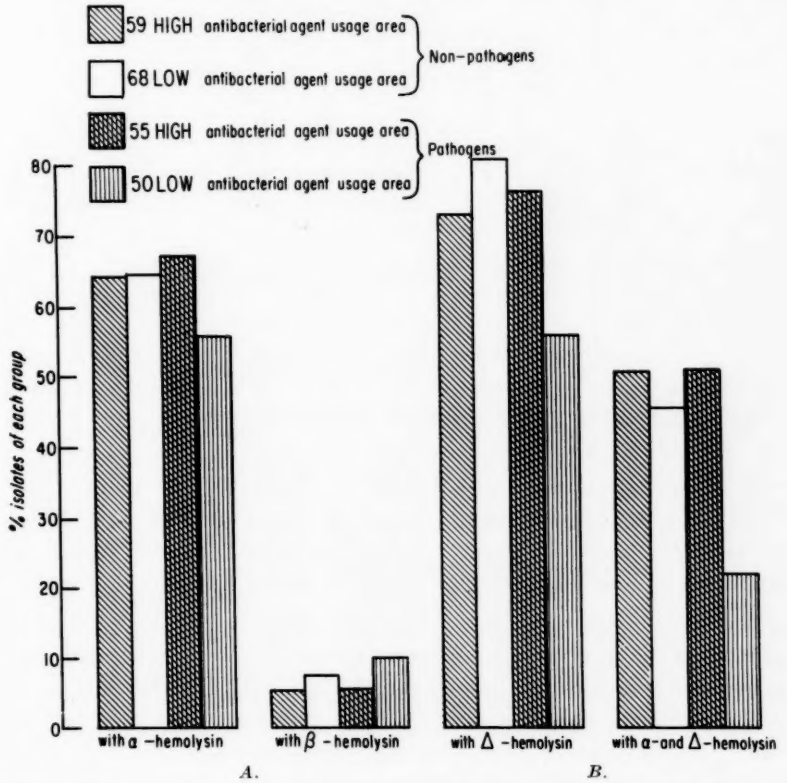


Fig. 1.—The frequency of elaboration of a particular hemolysin without regard to simultaneous production of other hemolysin(s) and the frequency of coincidence of α- with Δ-hemolysin is presented as a per cent of the tellurite-positive staphylococcal isolates comprising the 4 groups: *A*, nonpathogens from *high* and *low* antibacterial agent environments; *B*, pathogens from *high* and *low* antibacterial agent environments.

areas—a difference entirely due to the markedly greater frequency of occurrence of 2 hemolysins among pathogenic staphylococci from *high* usage areas. While individual hemolysins did not occur in significantly different frequency among the 4 groups of isolates, the simultaneous occurrence of α- and Δ-hemolysin elaboration was significantly more frequent with pathogens from *high* antibacterial agent areas.

Staphylokinase and staphyloprotease activities were not significantly different in occurrence among the 4 groups of staphylococcal isolates studied (Fig. 3).

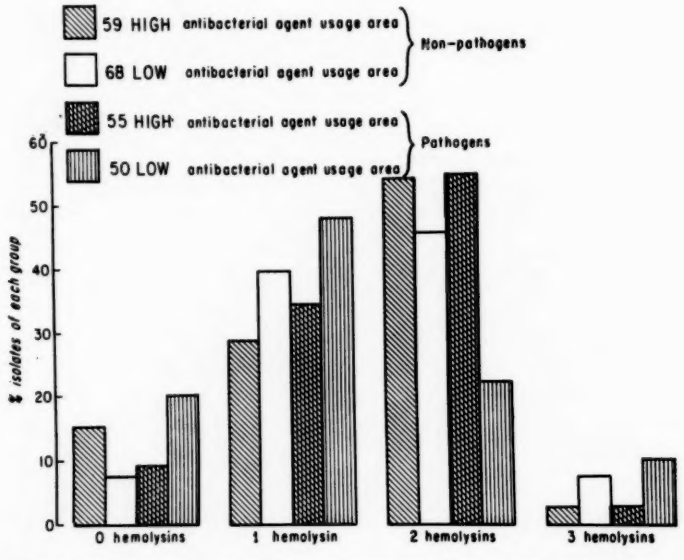


Fig. 2.—Nonpathogenic and pathogenic tellurite-positive staphylococci from *high* and *low* antibacterial agent environments displayed qualitative variation in hemolysin elaboration. The per cent of the isolates of each of the 4 groups is plotted according to number of different hemolysins produced.

Antiserum Reactivity.—Whether nonpathogens and pathogens were pooled or considered separately for statistical treatment, staphylococcal isolates from *high* and *low* antibacterial agent environments displayed no significant differences in antiserum reactivity—over-all or in terms of degree of reactivity (numbers of visible lines of interaction). However, nonpathogens from both *high* and *low* antibacterial agent environments accomplished visible reaction with commercial antistaphylococcal antiserum more frequently than did pathogens of corresponding antibacterial agent environments (Figs. 3 and 4).

Antibacterial Agent Susceptibility.—Over-all, as many isolates from *high* antibacterial agent environments were susceptible to as many of the 15 antibacterial agents tested as were isolates from *low* antibacterial agent–usage areas (Table III). However, when susceptibility is viewed agent by agent (Fig. 5 and Table IV) differences between staphylococcal isolates from *high* and *low* antibacterial agent environments become evident.

Penicillin.—Fifty units of penicillin per milliliter adversely affected 218 of the 232 staphylococcal isolates which were studied. Comparison of over-all

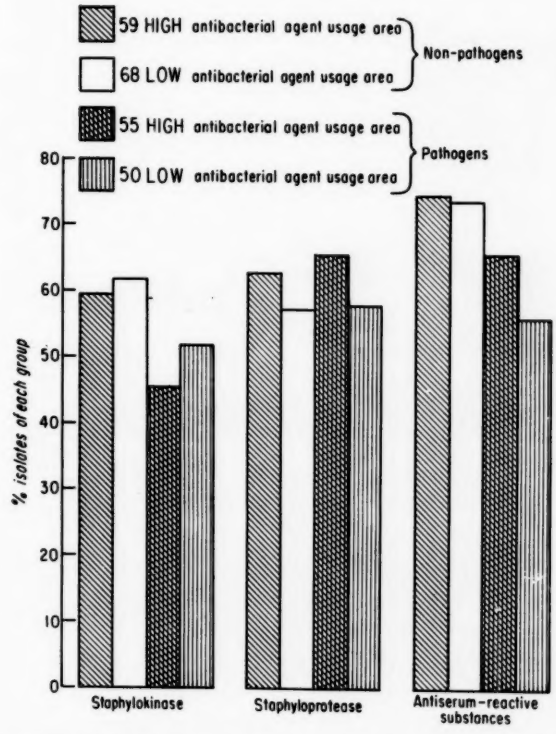


Fig. 3.—The frequency of elaboration of staphylokinase, staphyloprotease, and antiserum-reactive substance(s) is presented by plotting the per cent of the tellurite-positive staphylococcal isolates of each of the 4 groups (nonpathogens and pathogens from *high* and *low* antibacterial agent environments) which accomplished production of these substances.

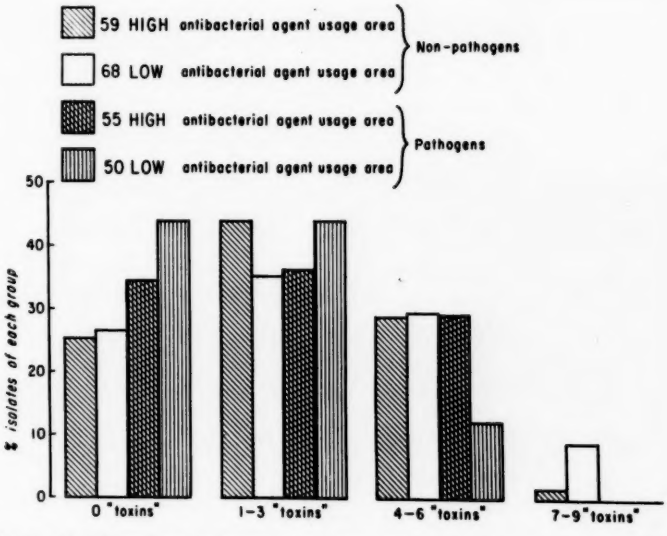
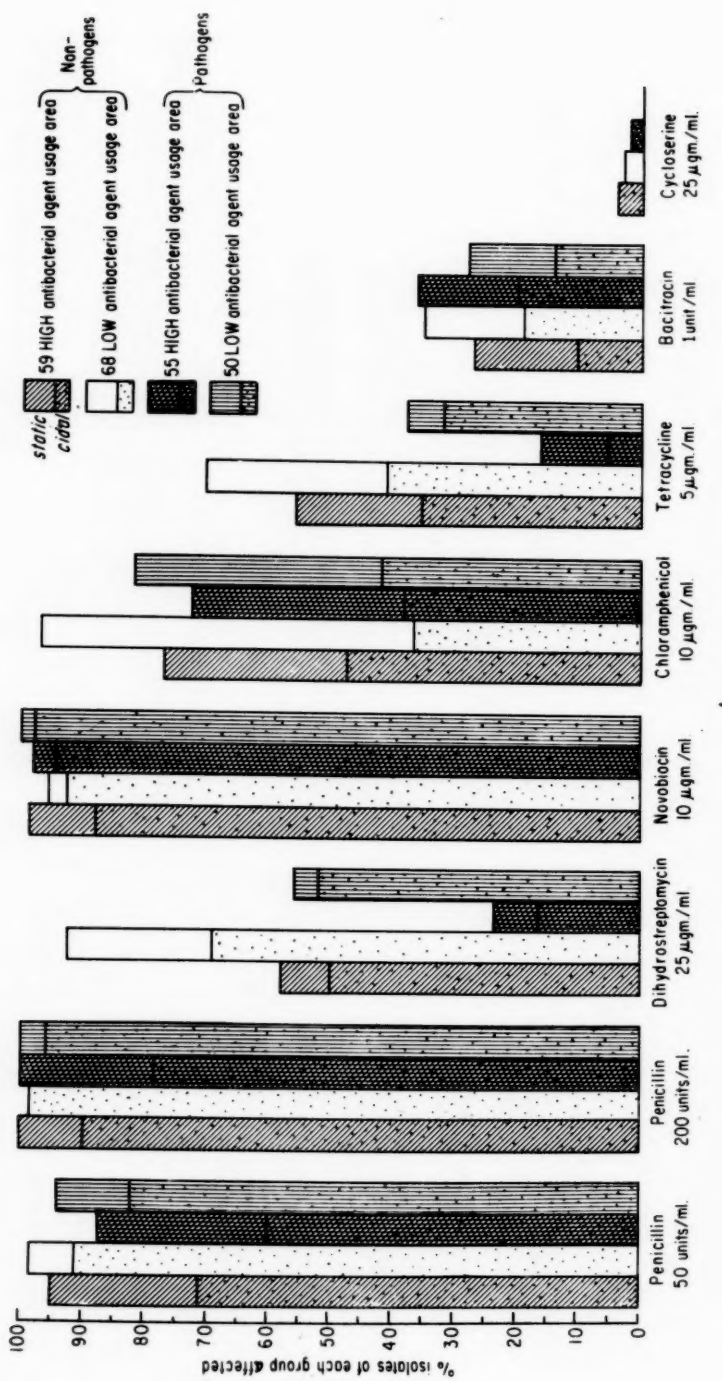
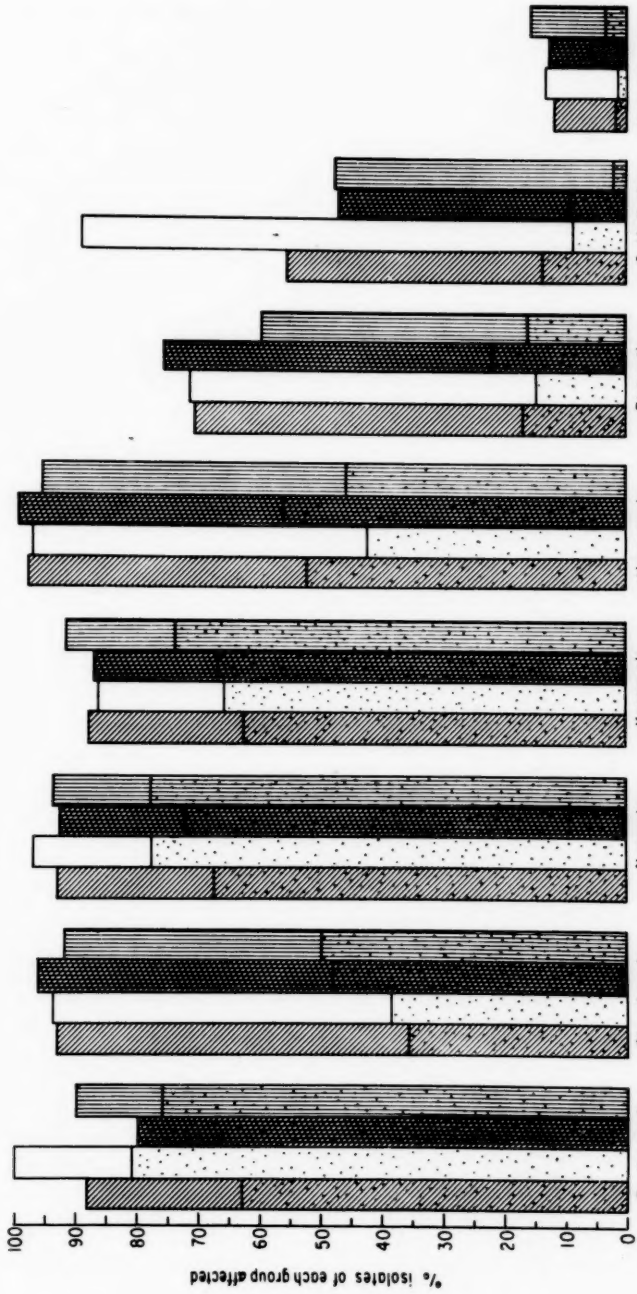


Fig. 4.—Lines of interaction between agar-diffusible products of nonpathogenic and pathogenic tellurite-positive staphylococcal isolates from *high* and *low* antibacterial agent environments and commercial, "polyvalent" rabbit and antistaphylococcal antiserum are referred to as "toxins." The height of the columns corresponds to the per cent of the staphylococci of each group which elaborated "toxins" to the extent indicated by the categories along the abscissa.


59 HIGH antibacterial agent usage area
68 LOW antibacterial agent usage area
55 HIGH antibacterial agent usage area
50 LOW antibacterial agent usage area
Non-pathogens
Pathogens
% isolates of each group affected
100
90
80
70
60
50
40
30
20
10
0
Penicillin 50 units/ml.
Penicillin 200 units/ml.
Dihydrostreptomycin 25 µgm./ml
Novobiocin 10 µgm./ml
Chloramphenicol 10 µgm./ml
Tetracycline 5µgm./ml
Bacitracin 1 unit/ml
Cycloserine 25 µgm./ml.

A.



B.

Fig. 5.—The per cent of the tellurite-positive nonpathogenic and pathogenic staphylococcal isolates from *high* and *low* antibacterial agent environments which were affected by a particular antibacterial agent are related to that agent. The test concentration of each agent is indicated. The over-all height of each column corresponds to the per cent affected; the stippled portion of each column indicates the per cent of staphylococcal isolates in that group which suffered a lethal effect under the test conditions; the unstippled remainder gives, by difference, the per cent of isolates inhibited from growth but not killed.

TABLE IV. EFFECT ON 232 CLINICAL ISOLATES OF TELLURITE-POSITIVE STAPHYLOCOCCI OF EACH OF 15 ANTIBACTERIAL AGENTS ASSESSED BY CHI-SQUARE COMPARISON OF ISOLATES* INHIBITED (BACTERIOSTATIC PLUS BACTERICIDAL EFFECT) FROM GROWTH, AND KILLED (BACTERICIDAL EFFECT)

AGENT	μG OR UNITS PER ML.	ISOLATE GROUPS ACCORDING TO ANTIBACTERIAL AGENT USAGE IN ENVIRONMENT OF ISOLATION					
		POOLED NONPATHOGENS WITH PATHOGENS: 114 HIGH VS. 118 LOW		NONPATHOGENS ALONE: 59 HIGH VS. 68 LOW		PATHOGENS ALONE: 55 HIGH VS. 50 LOW	
		INHIBITORY	BACTERICIDAL	INHIBITORY	BACTERICIDAL	INHIBITORY	BACTERICIDAL
Penicillin	50 units per ml.	No†	Yes‡	No†	Yes‡	No†	Yes‡
	200 units per ml.	No	Yes	No	No	No	Yes
Dihydrostreptomycin	25 μg	Yes	Yes	Yes	No	Yes	Yes
Novobiocin	10 μg	No	No	No	No	No	No
Chloramphenicol	10 μg	Yes	No	Yes	No	No	No
Tetracycline	5 μg	Yes	Yes	Yes	No	Yes	Yes
Bacitracin	1 unit per ml.	No	No	No	No	No	No
Cyloserine	25 μg	No	No	No	No	No	No
Erythromycin	10 μg	Yes	No	Yes	No	No	No
Oleandomycin	5 μg	No	No	No	No	No	No
Neomycin	10 μg	No	No	No	No	No	No
Kanamycin	15 μg	No	No	No	No	No	No
Vancomycin	5 μg	No	No	No	No	No	No
Ristocetin	5 μg	No	No	No	No	No	No
Sulfadiazine	150 μg	Yes	No	Yes	No	No	No
Nitrofurantoin	25 μg	No	No	No	No	No	No

*Grouped according to antibacterial agent usage in environment of isolation.
†"No" indicates $p>0.02$—lack of significant difference.
‡"Yes" indicates $p<0.02$, taken as a significant difference.

frequency of inhibition after these isolates were grouped according to antibacterial agent–usage environment revealed no statistically significant differences. However, 50 units of penicillin per milliliter was significantly more often bactericidal in effect on staphylococci from *low* antibacterial agent–usage areas (both nonpathogens and pathogens) than on staphylococci isolated from *high* antibacterial agent–usage areas. With 200 units of penicillin per milliliter, the same pattern of effectiveness was apparent among the 231 (of 232) isolates affected.

Dihydrostreptomycin.—Of 232 staphylococcal isolates, 138 were susceptible to 25 μg dihydrostreptomycin per milliliter. Both nonpathogens and pathogens from *low* antibacterial agent areas were significantly more often susceptible than were staphylococci isolated from *high* antibacterial agent environments. Dihydrostreptomycin was bactericidal in effect on 111 of the 138 isolates susceptible to this agent.

Novobiocin.—No significant differences in susceptibility to 10 μg novobiocin per milliliter were found in comparison of staphylococci isolated from *high* antibacterial agent environments with isolates from areas *low* in antibacterial agent usage. Of the 232 isolates tested, 229 were inhibited by novobiocin; of these, 215 were killed.

Chloramphenicol.—One hundred ninety-three (of 232) isolates were inhibited by 10 μg chloramphenicol per milliliter. Nonpathogenic staphylococci isolated from *low* antibacterial agent environments were significantly more often susceptible than were nonpathogenic isolates from *high* antibacterial agent–usage areas. A bactericidal effect was exhibited as often with isolates of one group as another.

Tetracycline.—In terms of both over-all and bactericidal effect, significantly more of the 109 staphylococcal isolates which were inhibited by 5 μg tetracycline per milliliter were obtained from areas of *low* antibacterial agent usage than were obtained from *high* usage areas. This contrast was most marked with pathogens.

Chloramphenicol and Tetracycline.—Chloramphenicol was significantly more often effective than tetracycline against nonpathogenic and pathogenic staphylococci from both *high* and *low* antibacterial agent environments. With isolates from *high* antibacterial agent areas, chloramphenicol was also significantly more often bactericidal in effect than was tetracycline. Fig. 6 relates all 232 isolates in a strain by strain correlation of susceptibility to chloramphenicol and/or tetracycline. There were 28 of 232 isolates resistant to both agents; 98 of 232 susceptible to both; 95 (49.7 per cent) of the 193 chloramphenicol-susceptible isolates were tetracycline-resistant, and 11 (10.1 per cent) of the 109 susceptible to tetracycline were at the same time chloramphenicol resistant.

Bacitracin.—Only 74 of the 232 staphylococcal isolates studied were affected by 1 unit bacitracin per milliliter. There were no significant differences

in susceptibility among the test staphylococci when grouped according to antibacterial agent–usage environment or pathogenicity.

Cycloserine.—All but 5 of the 232 staphylococcal isolates exposed to 25 μg of cycloserine per milliliter were insusceptible to this agent.

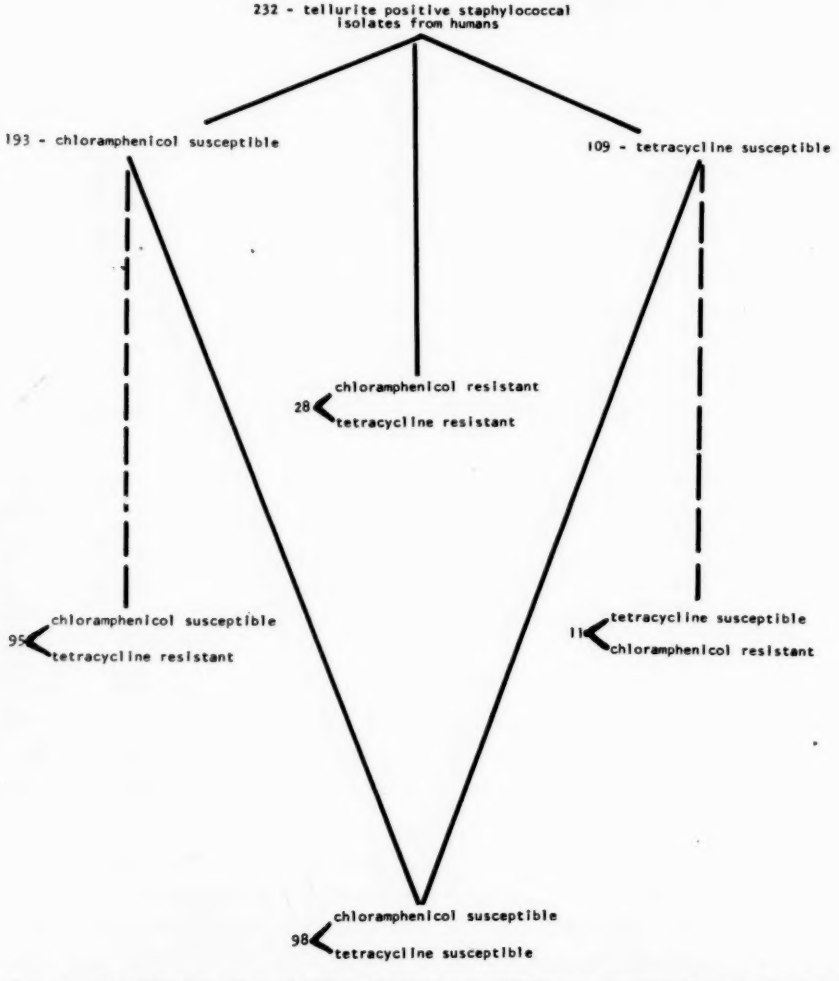


Fig. 6.—The effect of 10 μg chloramphenicol per milliliter is compared with the effect of 5 μg tetracycline per milliliter by noting the strain by strain susceptibility to these agents of 232 tellurite-positive staphylococcal isolates.

Erythromycin.—Of the 232 staphylococcal isolates tested, 209 were affected by 10 μg erythromycin per milliliter—a bactericidal effect in 171 instances. Nonpathogens from areas of *low* antibacterial agent usage were significantly more often susceptible than were nonpathogens from *high* usage environments.

Oleandomycin.—Susceptibility to 5 μg oleandomycin per milliliter was displayed by 218 of the 232 staphylococcal isolates tested. The data indicate there were no significant differences in susceptibility between the various groups of

isolates, which were assembled according to either environment from which isolated or pathogenicity.

Erythromycin and Oleandomycin.—As tested, erythromycin and oleandomycin did not display identity of effect on the 232 staphylococcal isolates examined. Erythromycin significantly more often exerted a bactericidal effect, although in terms of over-all inhibitory effect these agents were not significantly different. The relationships between erythromycin and oleandomycin effect, as gleaned from isolates by isolate comparison, is depicted in Fig. 7. While 201 of 232 isolates were susceptible to both agents and 6 of 232 were resistant to both, of the isolates susceptible to erythromycin, 8 (3.8 per cent) were resistant to oleandomycin. On the other hand, of the 218 oleandomycin-susceptible staphylococcal isolates, 17 (7.8 per cent) were erythromycin-resistant.

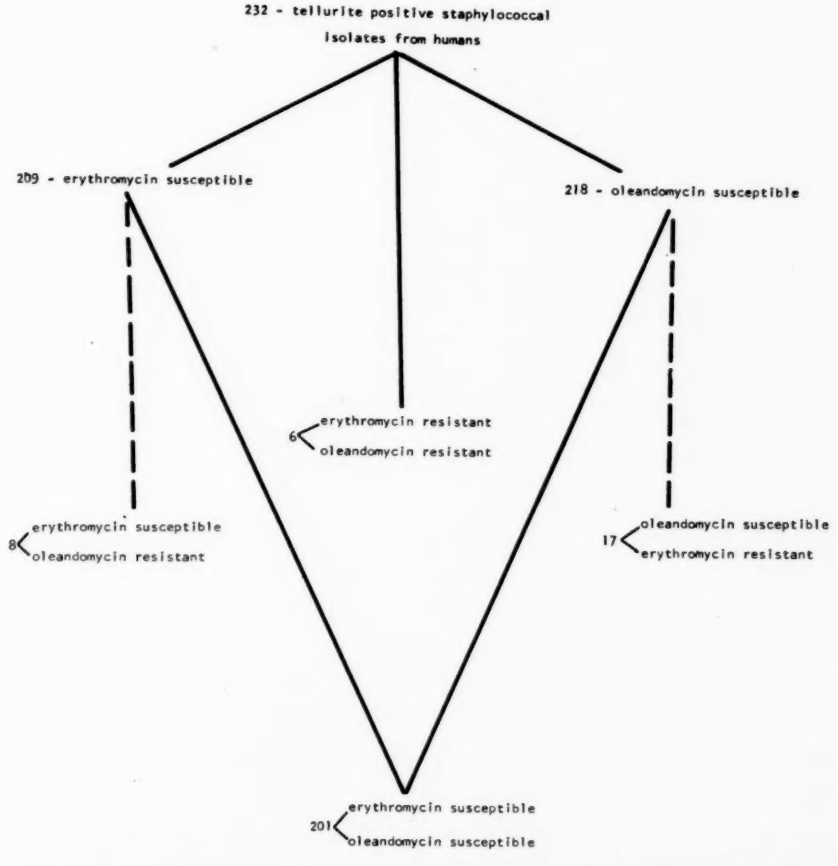


Fig. 7.—In terms of over-all inhibitor effectiveness, 10 μg of erythromycin per milliliter was not significantly more or less effective than 5 μg oleandomycin per milliliter, according to strain by strain comparison. Cross-resistance and susceptibility were virtually complete.

Neomycin.—There were 219 of the 232 staphylococcal isolates examined which were inhibited by 10 μg neomycin per milliliter; the effect was bactericidal

with 172 of the 219 susceptible strains. Comparison made after the staphylococci were grouped according to antibacterial agent usage area of isolation and/or pathogenicity failed to reveal significant differences in neomycin susceptibility.

Kanamycin.—Two hundred five of 232 isolates of staphylococcus were susceptible to 15 μg of kanamycin per milliliter; 156 of 205 were killed. Comparison of various groupings of the staphylococci tested disclosed no significant differences in susceptibility to this agent.

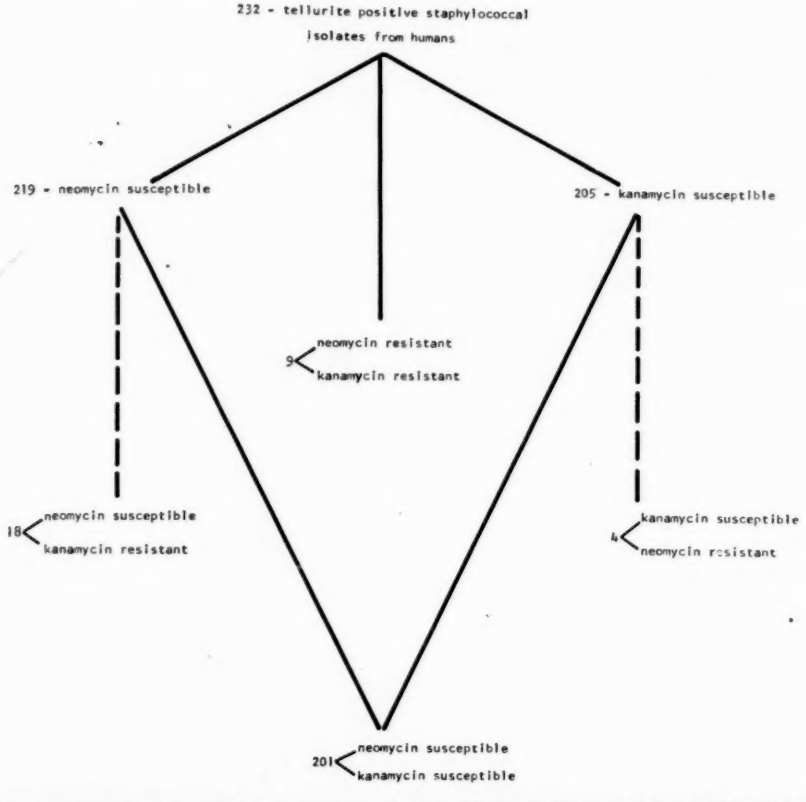

232 - tellurite positive staphylococcal isolates from humans
219 - neomycin susceptible
205 - kanamycin susceptible
9 neomycin resistant kanamycin resistant
18 neomycin susceptible kanamycin resistant
4 kanamycin susceptible neomycin resistant
201 neomycin susceptible kanamycin susceptible

Fig. 8.—Isolate by isolate comparison of the effect of 10 μg of neomycin per milliliter with the effect of 15 μg of kanamycin per milliliter disclosed no significant differences in the over-all susceptibility of 232 tellurite-positive staphylococci to these agents. Moreover, in the test situation employed, cross-resistance and susceptibility were virtually complete.

Neomycin and Kanamycin.—There were no significant differences in effect of neomycin and kanamycin under the conditions of assay employed in this study. Only 9 of 232 isolates were resistant to both neomycin and kanamycin, while 201 of 232 were susceptible to both drugs. Of the 219 neomycin-susceptible staphylococci, 18 (8.2 per cent) were at the same time resistant to kanamycin; of 205 kanamycin-susceptible isolates, 4 (1.9 per cent) were also resistant to neomycin (Fig. 8).

Vancomycin.—Applied in a concentration of 5 μg per milliliter, vancomycin was inhibitory to 227 of 232 staphylococcal isolates which were tested. To 114

of the 227 susceptible isolates, the effect was bactericidal. The extent to which antibacterial agents were used in the environment of isolation had no correlation with susceptibility to vancomycin.

Ristocetin.—In a concentration of 5 μg per milliliter, ristocetin prevented growth of 159 of the 232 isolates of staphylococcus tested; 40 of the 162 isolates were killed. There were no statistically significant differences in susceptibility in comparison of isolates obtained from environments of *high* with those from *low* antibacterial agent–usage areas.

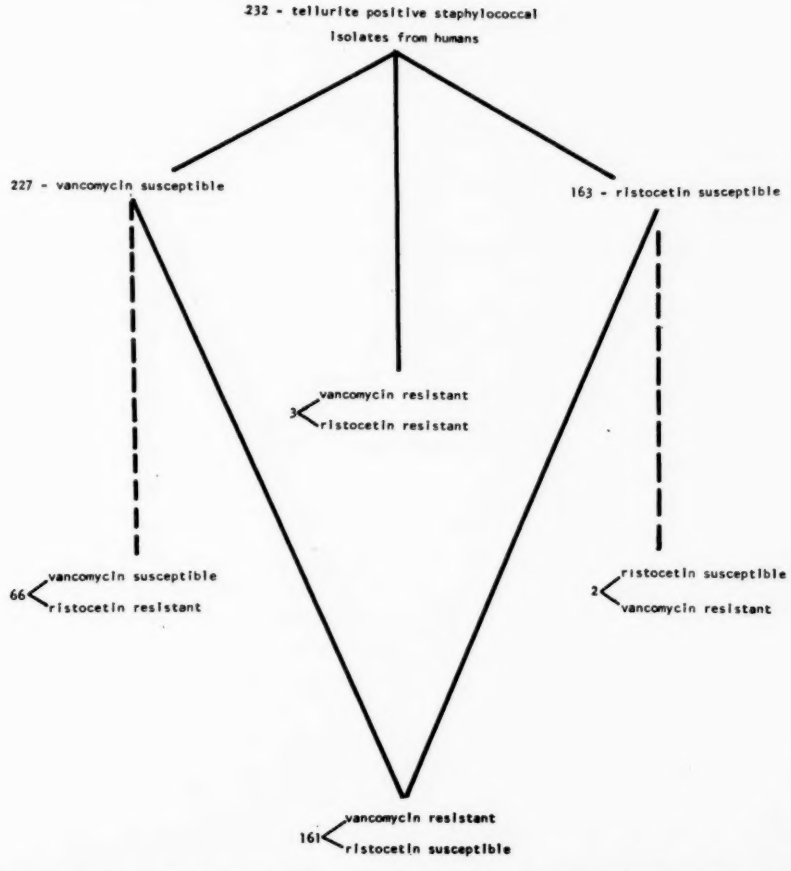


Fig. 9.—Vancomycin and ristocetin were both tested at a concentration of 5 μg per milliliter. Vancomycin was clearly more effective against the 232 tellurite-positive staphylococci investigated; only 2 of the 5 vancomycin-resistant isolates were also susceptible to ristocetin, whereas 66 of the 69 ristocetin-resistant isolates were at the same time vancomycin-susceptible.

Vancomycin and Ristocetin.—Vancomycin was uniformly more often effective against the test staphylococci of all groups than was ristocetin. There were 3 isolates of the 232 tested which were unaffected by either vancomycin or ristocetin and 157 which were susceptible to both these agents. With 70 of 227 (30.9 per cent) isolates, vancomycin was effective while ristocetin was not; 2 of 159 (1.3 per cent) isolates were at the same time ristocetin-susceptible and vancomycin-resistant (Fig. 9).

TABLE V. OF 232 NONPATHOGENIC AND PATHOGENIC TELLURITE-POSITIVE STAPHYLOCOCCAL ISOLATES FROM ENVIRONMENTS OF HIGH AND LOW ANTIBACTERIAL AGENT USAGE, 106 WERE PHAGE TYPABLE USING 25 BACTERIOPHAGES

PHAGE PATTERN	ANTIBACTERIAL AGENT ENVIRONMENT			
	HIGH (114 ISOLATES)		LOW (118 ISOLATES)	
	NONPATHOGENS (59 ISOLATES)	PATHOGENS (55 ISOLATES)	NONPATHOGENS (68 ISOLATES)	PATHOGENS (50 ISOLATES)
80	1		1	
29		1		
3a	1	2		
3c			1	1
7			1	1
42b	5	1	5	3
44a		1		
47c	2		2	
73	1		1	2
75			1	
77			1	1
81		6		2
80/81	1	1		2
52/80			1	
52a/81		1		
52a/70			1	
3a/3c				1
32/29			1	
3a/54		1		
3c/42b			1	
3c/55			1	1
7/42e	1			1
42b/81				1
54/81		1		
42b/52/80			1	
42d/80/81				1
52/52a/79		1		
53/54/73		1		
54/73/75		2		
3a/3c/47c		2		
3a/29/44a		1		
3a/80/81		1		
3b/3c/55			1	
7/42b/70			1	
42b/52a/79	1			
42b/54/73	1			
42b/80/81		1		
42d/47c/54	1			
54/70/73	1			
54/73/75			1	
42b/52/80/81	1			
52a/79/80/81	1			
3a/3b/3c/47c			1	
3a/3c/47c/55				1
3a/29/44a/42e		1		
42b/42e/54/70			1	
3a/29/44a/80/81		1		
3a/29/42b/44a/52			1	
3a/29/44a/52/54			1	
3c/70/73/75/77			1	
6/42b/53/70/73	1			
6/53/54/73/75			1	
42b/47/54/70/75	1			
42b/54/70/73/75		1		
3a/29/44a/52/52a/54			1	
3a/42e/47/70/73/77	1			
7/42b/42e/54/70/75				1
42b/44a/47/52/54/70			1	

TABLE V.—CONT'D

PHAGE PATTERN	ANTIBACTERIAL AGENT ENVIRONMENT			
	HIGH (114 ISOLATES)		LOW (118 ISOLATES)	
	NONPATHOGENS (59 ISOLATES)	PATHOGENS (55 ISOLATES)	NONPATHOGENS (68 ISOLATES)	PATHOGENS (50 ISOLATES)
42b/42e/54/70/73/VA4	1			
42b/54/55/70/73/75	1			
3a/29/42a/44a/52/52a/54				1
3a/42b/29/52/73/75/79/80/81				1
6/42b/42e/47/53/70/73/77/VA4				1
6/42b/42e/47e/54/73/75/77/VA4		1		
3a/7/29/44a/52/52a/53/54/80/81			1	
6/7/42b/42e/47/54/70/73/75/77/VA4		1		
Total	24	27	32	23

Sulfadiazine.—Growth of 144 of 232 staphylococcal isolates in broth free of p-aminobenzoic acid was inhibited by 150 μg sulfadiazine per milliliter. Significantly more nonpathogenic isolates from *low* than *high* antibacterial agent–usage areas were affected.

Nitrofurantoin.—There were 31 of 232 staphylococcal isolates which were susceptible to 10 μg nitrofurantoin per milliliter; of these 31, just 8 were killed by this agent under the test conditions employed. The susceptible strains were isolated as often from *high* as from *low* antibacterial agent–usage areas.

Bacteriophage Susceptibility.—One hundred six (45.7 per cent) of the 232 staphylococcal isolates examined were susceptible to one or more of 25 bacteriophages (Table V). The frequency of successful typing was not variable to a significant degree among isolates grouped according to antibacterial agent usage in the environment of isolation (Table VI). Those isolates which were phage typable were also considered with regard to susceptibility to Phages 80 and/or 81; while more isolates from areas of *high* antibacterial agent usage were lysed by these phages, singly or in combinations, the differences were not of statistical significance (Table VI).

When the 106 phage-susceptible staphylococcal isolates are regarded from the point of view of the nature of their human association (rather than kind of antibacterial agent environment from which isolation was accomplished) nonpathogens were not significantly more or less often phage typable than pathogens (Table VII). However, lysis by Phages 80 and/or 81 did occur in significantly decreased frequency among nonpathogens as compared with pathogens, particularly in *high* antibacterial agent–usage areas (Table VII).

From these data it is apparent that human associated staphylococci from environments *high* in content of antibacterial agents differed significantly from staphylococci isolated from humans in areas *low* in content of antibacterial agents by greater frequency of: (1) elaboration of 2 hemolysins (an α-, Δ-hemolysin combination in pathogens), (2) resistance to the bactericidal action(s) of penicillin, and (3) resistance to the action(s) of dihydrostreptomycin, chloramphenicol, tetracycline, erythromycin, and sulfadiazine. Staphylococci

from *high* antibacterial agent environments were not significantly different from those isolated from humans of environments *low* in content of antibacterial agents with regard to staphylokinase and staphyloprotease elaboration or antiserum reactivity; in susceptibility to novobiocin, bacitracin, cycloserine, oleandomycin, neomycin, kanamycin, vancomycin, ristocetin, or nitrofurantoin; or in susceptibility to 25 standard typing phages.

TABLE VI. COMPARISON OF BACTERIOPHAGE (25 STANDARD PHAGES) SUSCEPTIBILITY OF NONPATHOGENIC AND PATHOGENIC TELLURITE-POSITIVE STAPHYLOCOCCAL ISOLATES ACCORDING TO ANTIBACTERIAL AGENT ENVIRONMENT OF ISOLATION

ISOLATE GROUPS	PHAGE SUSCEPTIBLE	LYSED BY PHAGES 80 AND/OR 81 SINGLY OR IN COMBINATIONS
Pooled nonpathogens with pathogens according to environment:		
114 high 118 low	51/55 $0.80 > p > 0.75$	17/51 11/55 $0.20 > p > 0.10$
Nonpathogens according to environment:		
59 high 68 low	24/32 $0.50 > p > 0.30$	4/24 4/32 $0.70 > p > 0.20$
Pathogens according to environment:		
55 high 50 low	27/23 $0.50 > p > 0.30$	13/27 7/23 $0.25 > p > 0.20$

TABLE VII. COMPARISON OF BACTERIOPHAGE (25 STANDARD PHAGES) SUSCEPTIBILITY OF TELLURITE-POSITIVE STAPHYLOCOCCAL ISOLATES FROM HIGH AND LOW ANTIBACTERIAL AGENT–USAGE ENVIRONMENT ACCORDING TO PATHOGENICITY

ISOLATE GROUPS	PHAGE SUSCEPTIBLE	LYSED BY PHAGES 80 AND/OR 81 SINGLY OR IN COMBINATIONS
High and *low* antibacterial agent environment isolates according to pathogenicity:		
127 nonpathogens 105 pathogens	56/50 $0.70 > p > 0.50$	8/56 20/50 $0.005 > p > 0.001$
High antibacterial agent environment isolates according to pathogenicity:		
59 nonpathogens 55 pathogens	24/27 $0.50 > p > 0.30$	4/24 13/27 $0.027 > p > 0.01$
Low antibacterial agent environment isolates according to pathogenicity:		
68 nonpathogens 50 pathogens	32/23 $0.95 > p > 0.90$	4/32 7/23 $0.20 > p > 0.10$

Pathogens from *high* antibacterial agent–usage areas were significantly more often susceptible to Phages 80 and/or 81, singly or in combinations, than were: (1) nonpathogens isolated from similar (in antibacterial agent content) environments, (2) pathogens or nonpathogens of *low* antibacterial agent–usage areas.

DISCUSSION

Nearly all staphylococci which are tellurite positive–coagulase positive, whether found in nasal colonies or in the pus of lesions, have a broad and varied metabolic endowment.[8, 15] This generalization is not overthrown when human associated staphylococci garnered in one community during a given span of time are grouped according to antibacterial agent content of the environment of isolation. Elek and Levy[12] found that pathogenic (more often than nonpathogenic) staphylococci elaborate multiple hemolysins—usually in an α- and Δ-hemolysin combination. The data presented above indicate that an α-, Δ-hemolysin pattern was found with significantly increased frequency among pathogens from *high* antibacterial agent–usage areas; pathogens from *low* antibacterial agent–usage areas were not similarly well endowed with α-, Δ-hemolysins, a difference for which no explanation is at this time apparent. There is no obvious metabolic relationship between capacity to elaborate α- and/or Δ-hemolysins and survival in the presence of antibacterial agents. It may be that the combination of increased frequency of α-, Δ-hemolysin elaboration with increased frequency of resistance to antibacterial agents (properties which set off those staphylococci isolated from *high* antibacterial agent–usage areas) is a fortuitous imposition of the criteria set up to delineate these groups of staphylococcal isolates. Whatever the explanation, α-, Δ-hemolysin elaboration was the one exception to an otherwise striking lack of change in enzyme-mediated attributes of staphylococcus concomitant with the observed inverse variation in susceptibility to antibacterial agents present in the environment.

Hinton and Orr[14] noted α-, Δ-hemolysin production was commonly a property of pathogenic staphylococci and reported that staphylokinase was elaborated with significantly greater frequency by pathogens and by intrahospital carrier strains than by extrahospital carrier strains of coagulase-positive staphylococci. Elek[8] found no differences between lesion and carrier isolates of coagulase-positive staphylococci with regard to frequency of staphylokinase production. Our data are in keeping with those of Elek—production of staphylokinase, as well as staphyloprotease, was an accomplishment exhibited as often by colonizing tellurite-positive staphylococci as by lesion-causing tellurite-positive staphylococci.

The five groups of staphylococcal isolates studied by Hinton and Orr[14] were arranged by these authors in order of decreasing ability to elaborate agar diffusible antiserum reactants: I, closed infection isolates (primary lesions such as carbuncles) significantly $>$ II, open infection isolates (wounds, burns, ulcers) and IV, carrier isolates from hospital personnel; II and IV were significantly $>$ III, patient carrier isolates, and V, extrahospital carrier isolates. In apparent contrast, there were no significant differences among the four groups of staphylococci of the present report with regard to elaboration of agar-diffusible antiserum reactants. There are at least 2 factors worthy of mention in regard to this disagreement: (1) the criteria used in grouping the staphylococci of each study were not identical; and (2) the definition of "significant" varied—$p < 0.50$ according to Hinton and Orr; $p \leq 0.02$ in the present study.

The commonly used antibacterial agents prescribed in 1959 for systemic therapy in the Salt Lake County General Hospital (the major *high* antibacterial agent environment from which staphylococci were obtained for study) were as follows: penicillin, chloramphenicol, tetracyclines, streptomycin-dihydrostreptomycin, erythromycin, sulfonamides, and nitrofurantoin (Table II). Novobiocin, bacitracin, cycloserine, neomycin, kanamycin, oleandomycin, vancomycin, and ristocetin were either not employed or used only rarely in systemic therapy during the period when these staphylococci were collected for study. That resistance to the actions of penicillin, dihydrostreptomycin, chloramphenicol, tetracycline, erythromycin, and sulfadiazine occurred significantly more frequently among staphylococci from an environment in which these agents were in daily use merely reflects the survival value which resistance has to staphylococci bred in such an environment. Resistance to the actions of antibacterial agents not displayed in the environment from which the staphylococci were isolated was encountered but rarely. Of note is the fact that without provocation such resistance was not of significantly greater frequency of occurrence among those groups of staphylococci which were already possessed of powers of resistance to other commonly employed antibacterial agents than it was among staphylococci still susceptible to these commonly used agents. We should have learned nothing from our experience with staphylococcus since the use of penicillin became widespread if we expected anything less in the way of adaption from this eminently pliable bacterial species. A logical consequence attends the observation of even rare occurrence of resistance to agents not applied in therapy; namely, a minimum of 2 individually effective agents should be given whenever antistaphylococcal chemotherapy is indicated. This course has been suggested as a useful counter to the mutability of staphylococcus.[7, 20]

Quite reasonably, persons coming into an environment of *high* antibacterial agent content soon have their native, antibacterial agent–susceptible staphylococci supplanted with colonizing or disease-causing staphylococci of a kind which can survive in the presence of antibacterial agents.[21-23] Following return to a normal (*low* in content of antibacterial agents) environment, reversion of staphylococcal flora to a kind not resistant to antibacterial agents is the rule,[24-26] for there is no longer competitive advantage to the property of resistance to antibacterial agents when the environment is devoid of these agents. In short, to the extent humans dwell in environments which are *low* in content of antibacterial agents, to that extent will the staphylococci which associate with humans be susceptible to antibacterial agents. This prediction of lack of homogenization of human associated staphylococci is confirmed for the Salt Lake City community.

Reports of family unit staphylococcal infections due to "hospital antibiotic–resistant" staphylococci, apparently brought home from the hospital nursery by neonates,[27, 28] are seemingly in conflict with both the data of this study and their rationalization as given above. Yet, it must be remembered that when staphylococci which are brought home from the hospital by infants (or children or adults) cause illness in any member of the family unit, antibacterial agent

therapy is almost invariably instituted—a *high* antibacterial agent environment is perpetuated in the home. In addition, it is possible that the hospital strain of staphylococcus did not always hold sway to cause the illness in the home which was ascribed to it.

For example, Hurst and Grossman[28] reported the phage type of staphylococci isolated from infants in the home was either Type 80/81 (identical with the nursery type) or Type 80/81/52/52a, which was assumed to be mutant to the nursery Type 80/81. Identity of phage type is not of itself incontrovertible evidence of identity of source of staphylococci in terms of fission from a common staphylococcal ancestor; assuming an observed change in phage pattern is due to mutation compounds the risk of relying on circumstantial evidence.

More pertinent to the thesis of replacement of antibacterial agent–resistant staphylococci by antibacterial agent–susceptible staphylococci when the environment is changed to one virtually without antibacterial agents is the finding in this same study[28] that a few isolates (number not specified) obtained at home visit were susceptible to tetracyclines, or to tetracyclines and penicillin; the hospital nursery staphylococcus was resistant to tetracyclines and penicillin.

Although somewhat aside from the direct purpose of this study, the susceptibility data obtained permit interesting and useful comparison of certain pairs of antibacterial agents. Thus, chloramphenicol was significantly more often effective than tetracycline (Fig. 6) under the test conditions described.

Comparing the effect of 10 μg chloramphenicol per milliliter with 5 μg tetracycline per milliliter is reasonable from a clinical point of view since with dosage tolerable to patients chloramphenicol yields higher concentrations in the blood.[29] The greater effectiveness of chloramphenicol as compared with tetracycline in suppressing growth of staphylococci, noted several years ago, has been ascribed to the less frequent use of chloramphenicol in therapy.[30] This explanation has worn thin with time since in recent years chloramphenicol has again become widely used. Moreover, in 1959 more chloramphenicol than tetracyclines (tetracycline + congeners) was dispensed in the Salt Lake County General Hospital—the primary source of the *high* antibacterial agent environment staphylococcal isolates of this study. The suggestions put forth by Fisher[31] that staphylococci develop resistance to chloramphenicol both more slowly and less completely than to other agents and then more rapidly become again susceptible on withdrawal of chloramphenicol provide a more reasonable basis for explanation of the definitely greater antistaphylococcal effectiveness of chloramphenicol as compared with tetracycline.

Erythromycin was the first introduced and is the best known of a group of congeneric, macrolide antibiotics which includes carbomycin, spiramycin, and oleandomycin.[32] For therapy, erythromycin was not really challenged until preparation of triacetyloleandomycin resulted in a practical oral dosage form of oleandomycin.[33] Absorption of triacetyloleandomycin from the human gastrointestinal tract appears to be less efficient than is absorption of orally administered esterified (propionic acid) erythromycin.[34] For this reason, susceptibility data observed at 10 μg per milliliter for erythromycin and 5 μg

per milliliter for oleandomycin are proper for clinically significant comparison of these drugs. In this setting, the over-all superiority of erythromycin as an antistaphylococcal agent is quite clear since erythromycin significantly more often exerted a bactericidal effect. Cross-susceptibility and resistance were virtually complete in terms of over-all inhibitory effect.

Neomycin and kanamycin are optically active complex bases which display quite similar spectra of antibacterial action. Allegedly less toxic to humans, kanamycin is usually applied in higher dosage in systemic therapy than is neomycin; accordingly, these agents were compared, with regard to antistaphylococcal effectiveness, using 10 μg neomycin per milliliter and 15 μg kanamycin per milliliter. Under these test conditions, there were no significant differences in effect on the staphylococci examined, regardless of antibacterial agent content of environment of origin. These data do not prove that molecule for molecule neomycin is more active than kanamycin against staphylococci, as asserted by Feingold.[35] This may well be the case, however, for the test concentration of kanamycin was one-third greater than was that of neomycin.

Vancomycin and ristocetin, while distinct chemical entities, are both amphoteric substances of rather high molecular weight ($> 3{,}000$) and of similar antibacterial spectrum. They were compared at a concentration of 5 μg per milliliter, since both agents when given in the usual clinical dosage, yield blood concentrations of around 5 μg per milliliter.[29] Vancomycin was far more often effective, both over-all and in exerting a bactericidal effect, than was ristocetin.

As reviewed by Blair,[36] of coagulase-positive staphylococci isolated from extrahospital environments, about 70 per cent are susceptible to typing by means of bacteriophages. A variety of phage patterns in four phage groups have been observed. Coagulase-positive staphylococci obtained from hospital inpatients were more often susceptible to bacteriophages—typing may be successful with as many as 90 per cent of such strains. While patterns vary, the hospital, antibacterial agent–resistant staphylococci most often fall in Group III and next most often in Group I.

With the 232 tellurite-positive staphylococcal isolates considered in this report, there was less over-all success in phage typing. In addition, no significant differences in frequency of phage susceptibility were discernible in comparison of staphylococci grouped: according to extent of antibacterial agent usage in the area of isolation; nonpathogens with pathogens. The phage patterns observed were often untidy in not being clearly assignable to a classic phage group. However, pathogens of *high* antibacterial agent areas were significantly more often susceptible to Phages 80 and/or 81 singly or in combinations, a finding in keeping with other reports.[37] In other respects, bacteriophage typing was of no value in giving evidence for or against complete intracommunity admixture or potentially pathogenic staphylococci.

SUMMARY AND CONCLUSIONS

Two hundred thirty-two tellurite-positive isolates of human associated staphylococci cultured in 1958 and 1959 in Salt Lake City, Utah, were obtained

from environments *high* in content of antibacterial agents (59 nonpathogens; 55 pathogens) and *low* in content of antibacterial agents (68 nonpathogens; 50 pathogens).

These groups of staphylococcal isolates were compared with regard to certain enzymatic accomplishments (α-, β-, Δ-hemolysin, kinase and protease activities), antiserum reactivity, susceptibility to 15 antibacterial agents and 25 bacteriophages. Significant differences were observed, with regard to hemolysins elaborated, and susceptibilty to penicillin, dihydrostreptomycin, chloramphenicol, tetracycline, erythromycin, and sulfadiazine, which distinguished *high* from *low* antibacterial agent environment staphylococci. As recently as 1959, admixture of human associated staphylococci was not complete in Salt Lake City.

Comparison of in vitro susceptibility of 232 staphylococcal isolates to chloramphenicol-tetracycline, erythromycin-oleandomycin, neomycin-kanamycin, vancomycin-ristocetin indicated the first mentioned antibiotic of each pair was more effective than the last mentioned.

It is a pleasure to acknowledge the aid of Dr. John R. Waldo in the collection of nose cultures from high school seniors, Eugene Speakman (Chief Pharmacist, Salt Lake County Hospital) in the completion of Table IV, and the technical assistance of James N. Whitesides and Gloria Bristow. Garth F. Croft, Ingeborg E. Nelson (Clinical Microbiology Laboratory, Salt Lake County General Hospital), and Dr. Ralph A. Knight (Bacteriology Laboratory, Veterans Administration Hospital of Salt Lake City) isolated many of staphylococcal strains which were studied.

Addendum.—Since this study was completed, published reports (Bondi, A., Kornblum, J., and Forte, C.: Inhibition of Antibacterial Activity of Cycloserine by Alpha-Alanine, Proc. Soc. Exper. Biol. & Med. 96: 270, 1957; and Shockman, G. D.: Reversal of Cycloserine Inhibition of D-Alanine, Proc. Soc. Exper. Biol. & Med. 101: 693, 1959) have come to the author's attention which indicate that the potential antistaphylococcal effect of cycloserine may have been subverted by the presence of alpha-alanine in the culture medium.

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LABORATORY METHODS

BACTERIOPHAGE IMPREGNATED DISKS WITH A REDOX INDICATOR FOR RAPID TYPING OF RESISTANT STRAINS OF STAPHYLOCOCCUS AUREUS

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A rapid method for bacteriophage typing of staphylococci is described. The method is based upon the use of phage impregnated filter paper disks containing triphenyltetrazolium chloride. Since the reduction of this indicator to a colored formazan precedes the appearance of macroscopic growth, the results of the test may be read 4 to 6 hours after inoculation. Methods for preparing the test disks and results obtained when 40 strains of coagulase-positive staphylococci were typed by the disk technique are presented. Precautions to be observed and limitations of the disk system are discussed.

THE literature contains numerous reports of attempts to extend the flexibility and usefulness of the paper disk method for determining antibiograms of microorganisms by reducing the time required to obtain the results of sensitivity tests. Most of the "rapid" procedures are characterized by the addition of either natural or synthetic oxidation-reduction indicators to the test media. Hemoglobin of whole blood has been widely used as a color indicator in drug sensitivity tests.[1-6] Synthetic redox indicators such as the tetrazolium salts and resazurin have also been used with varying degrees of success in connection with the seeded agar plate–disk technique and in paper disk systems.[7-13]

The principle involved in the use of redox indicators is simple and involves the rapid visualization of areas of normal growth on the test plate in contrast to areas of growth inhibition produced by the test drugs or antibiotics. Since changes in color of a redox indicator precede the appearance of macroscopic growth, the results of sensitivity tests may be read within 6 to 12 hours after inoculation with accuracy.

In many cases, however, the additional work required to prepare special media and reagents for the rapid techniques has discouraged their adoption by clinical laboratories. Also, reports indicating that some of the synthetic

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redox indicators are toxic for microorganisms, especially the gram-positive bacteria, have delayed acceptance of the rapid techniques.[10-12]

In contrast to antibiotic sensitivity testing, there have been relatively few attempts to simplify bacteriophage typing procedures. Mora and Eisenstark[14] successfully adapted the paper disk technique to the differentiation of bacterial strains through the use of bacteriophages. The phage patterns of a number of strains of bacteria, including strains of *Staphylococcus aureus*, were rapidly and accurately determined through the use of paper disks impregnated with appropriate phages. Results were read directly from the test plates and were identical to those obtained by other investigators using conventional phage typing procedures. The phage impregnated disks were shown to be stable in storage at temperatures of 4° C., –10° C., and at room temperatures varying between 23° and 42° C. for periods of 90 days.

The addition of a redox indicator to paper disks impregnated with phage to produce a completely self-contained typing system was recently suggested.[15] It was shown that such a self-contained system was feasible and that the time required to determine the phage patterns of coagulase-positive strains of *S. aureus* was greatly reduced when phage disks were used. Since such disks could be mass produced, it was suggested that they be prepared, standardized, and distributed from a central laboratory. In this way all clinical laboratories now conducting antibiotic sensitivity tests could also conduct, simultaneously, phage typing of staphylococcal strains rapidly and with minimum expense.

This report contains information concerning the preparation of phage impregnated paper disks with a redox indicator and presents the results obtained when 40 strains of *S. aureus* isolated from clinical specimens were typed by the disk method. For comparative purposes the results obtained by the conventional procedure are also included.

MATERIALS AND METHODS

Bacteriophages.—The bacteriophages used in preparation of the disks were obtained from the Laboratory Divisions, Hospital for Joint Diseases, New York, through the courtesy of Dr. J. E. Blair. The 21 "basic" phages recommended by the International Committee and two additional phages currently being used in the United States were included.

Cultures.—Strains of staphylococci isolated during outbreaks of infections in hospitals were used in the preliminary tests of the phage disks. Also included were 15 strains isolated and typed by other laboratories. All strains were coagulase-positive and were maintained on trypticase soy agar slants. Prior to typing, the strains were transferred three times in trypticase soy broth as a result of the widespread use of topical ointments containing neomycin in the hospitals.[20]

Preparation of Disks.—Test disks were prepared according to the previously described method[15] with minor modifications. Sterile filter paper disks (S and S 740-E) measuring 12.7 mm. in diameter were used. The disks were first impregnated with 0.5 mg. of 2, 3, 5-triphenol tetrazolium chloride suspended in 0.2 ml. of sterile distilled water. The disks were then dried *in vacuo* over a drying agent for 3 days.

Phage suspensions containing approximately 5×10^7 particles per milliliter were prepared in trypticase soy broth. The broth contained 400 mg. of added calcium chloride per liter. Prior to impregnation purified ovalbumin was added to the adjusted phage suspensions to give a final concentration of 5 to 7 per cent. Impregnation of the disks was accomplished by adding 0.2 ml. (approximately 10^7 particles) of the adjusted suspensions to the disks with pipettes. After the addition of the phage the disks were dried rapidly *in vacuo* and

stored over a drying agent for 3 days. Successful preparation of typing disks depends to a large extent upon achieving a high degree of dehydration in the test system after impregnation with phage. Completed disks were stored in tightly sealed brown glass bottles over a drying agent.

Test Procedure.—The phage patterns of the test strains were obtained by preparing a slightly turbid (4 to 6 hours) broth culture in trypticase soy broth and inoculating each of the 24 disks representing the typing phages with 0.2 ml. of the broth. Appropriate control disks were included with each series. Trays containing the test systems were covered with moist towels to prevent evaporation and incubated at 37° C. until the indicator control disks showed maximum color development (4 to 6 hours). Results were obtained after incubation by recording the numbers corresponding to the disks that did not undergo a color change (white to pink).

Typing by the microdroplet procedure was conducted according to the technique described by Blair and Carr.[16]

TABLE I. PHAGE PATTERNS OF STAPHYLOCOCCAL STRAINS AS DETERMINED BY MICRODROP AND PHAGE DISK TECHNIQUES

STRAIN	PHAGE PATTERNS		
	MICRODROP	DISK (10^3 PARTICLES)	DISK (10^7 PARTICLES)
1	29/52/80/81	29/52/80	29/52/80/81
2	29/52/80/81	29/52/80	29/52/80/81
3	29/52/52A/80/81	29/52/52A/80/81	29/52/52A/80/81
4	29/52/79/80	29/52/79/80	29/52/79/80
5	47/54/77/VA4	47/54/77/VA4	47/54/77/VA4
6	47/53/54/77/VA4	47/53/77/VA4	47/53/54/77/VA4
7	53/VA4	53/VA4	53/VA4
8	6/47	Not tested	6/47
9	6/47	Not tested	6/47
10	6/47	Not tested	6/47
11	6/47	Not tested	6/47
12	6/47/81	Not tested	6/47/81
13	3A/3B/3C/71	3B/3C/71	3A/3B/3C/71
14	80/81	80/81	80/81
15	6/47/53/54/77/VA4	Not tested	6/47/53/54/77/VA4
16	52/52A/79/80	52/52A/79/80	52/52A/79/80
17	3A/3B/3C/71	3A/3B/3C/71	3A/3B/3C/71
18	79/53	79/53	79/53
19	3A/53	3A/53	3A/53
20	6/47/54/75/VA4	6/47/54/75/VA4	6/7/47/54/75/VA4
21	29/52/52A/80	29/52/52A/80	29/52/52A/80
22	52/80/81	52/80/81	52/80/81
23	42E/53/54	42E/53/54	42E/53/54
24	Untypable	Untypable	Untypable
25	42D/42E/52/77	Not tested	42D/42E/52/77

RESULTS

The phage patterns of 25 strains of coagulase-positive staphylococci isolated from the hospital environment and nasal cultures are presented in Table I. Table II shows the results obtained when 15 strains of staphylococci, previously typed by the conventional technique, were typed by the disk method. Results obtained by the microdroplet method and the reported patterns of the strains are presented in the tables for comparative purposes. The need for having an excess of phage particles incorporated into the disks is shown by the results obtained with disks containing approximately 10^3 particles.

redox indicators are toxic for microorganisms, especially the gram-positive bacteria, have delayed acceptance of the rapid techniques.[10-12]

In contrast to antibiotic sensitivity testing, there have been relatively few attempts to simplify bacteriophage typing procedures. Mora and Eisenstark[14] successfully adapted the paper disk technique to the differentiation of bacterial strains through the use of bacteriophages. The phage patterns of a number of strains of bacteria, including strains of *Staphylococcus aureus,* were rapidly and accurately determined through the use of paper disks impregnated with appropriate phages. Results were read directly from the test plates and were identical to those obtained by other investigators using conventional phage typing procedures. The phage impregnated disks were shown to be stable in storage at temperatures of 4° C., –10° C., and at room temperatures varying between 23° and 42° C. for periods of 90 days.

The addition of a redox indicator to paper disks impregnated with phage to produce a completely self-contained typing system was recently suggested.[15] It was shown that such a self-contained system was feasible and that the time required to determine the phage patterns of coagulase-positive strains of *S. aureus* was greatly reduced when phage disks were used. Since such disks could be mass produced, it was suggested that they be prepared, standardized, and distributed from a central laboratory. In this way all clinical laboratories now conducting antibiotic sensitivity tests could also conduct, simultaneously, phage typing of staphylococcal strains rapidly and with minimum expense.

This report contains information concerning the preparation of phage impregnated paper disks with a redox indicator and presents the results obtained when 40 strains of *S. aureus* isolated from clinical specimens were typed by the disk method. For comparative purposes the results obtained by the conventional procedure are also included.

MATERIALS AND METHODS

Bacteriophages.—The bacteriophages used in preparation of the disks were obtained from the Laboratory Divisions, Hospital for Joint Diseases, New York, through the courtesy of Dr. J. E. Blair. The 21 "basic" phages recommended by the International Committee and two additional phages currently being used in the United States were included.

Cultures.—Strains of staphylococci isolated during outbreaks of infections in hospitals were used in the preliminary tests of the phage disks. Also included were 15 strains isolated and typed by other laboratories. All strains were coagulase-positive and were maintained on trypticase soy agar slants. Prior to typing, the strains were transferred three times in trypticase soy broth as a result of the widespread use of topical ointments containing neomycin in the hospitals.[20]

Preparation of Disks.—Test disks were prepared according to the previously described method[15] with minor modifications. Sterile filter paper disks (S and S 740-E) measuring 12.7 mm. in diameter were used. The disks were first impregnated with 0.5 mg. of 2, 3, 5-triphenol tetrazolium chloride suspended in 0.2 ml. of sterile distilled water. The disks were then dried *in vacuo* over a drying agent for 3 days.

Phage suspensions containing approximately 5×10^7 particles per milliliter were prepared in trypticase soy broth. The broth contained 400 mg. of added calcium chloride per liter. Prior to impregnation purified ovalbumin was added to the adjusted phage suspensions to give a final concentration of 5 to 7 per cent. Impregnation of the disks was accomplished by adding 0.2 ml. (approximately 10^7 particles) of the adjusted suspensions to the disks with pipettes. After the addition of the phage the disks were dried rapidly *in vacuo* and

stored over a drying agent for 3 days. Successful preparation of typing disks depends to a large extent upon achieving a high degree of dehydration in the test system after impregnation with phage. Completed disks were stored in tightly sealed brown glass bottles over a drying agent.

Test Procedure.—The phage patterns of the test strains were obtained by preparing a slightly turbid (4 to 6 hours) broth culture in trypticase soy broth and inoculating each of the 24 disks representing the typing phages with 0.2 ml. of the broth. Appropriate control disks were included with each series. Trays containing the test systems were covered with moist towels to prevent evaporation and incubated at 37° C. until the indicator control disks showed maximum color development (4 to 6 hours). Results were obtained after incubation by recording the numbers corresponding to the disks that did not undergo a color change (white to pink).

Typing by the microdroplet procedure was conducted according to the technique described by Blair and Carr.[16]

TABLE I. PHAGE PATTERNS OF STAPHYLOCOCCAL STRAINS AS DETERMINED BY MICRODROP AND PHAGE DISK TECHNIQUES

STRAIN	PHAGE PATTERNS		
	MICRODROP	DISK (10^3 PARTICLES)	DISK (10^7 PARTICLES)
1	29/52/80/81	29/52/80	29/52/80/81
2	29/52/80/81	29/52/80	29/52/80/81
3	29/52/52A/80/81	29/52/52A/80/81	29/52/52A/80/81
4	29/52/79/80	29/52/79/80	29/52/79/80
5	47/54/77/VA4	47/54/77/VA4	47/54/77/VA4
6	47/53/54/77/VA4	47/53/77/VA4	47/53/54/77/VA4
7	53/VA4	53/VA4	53/VA4
8	6/47	Not tested	6/47
9	6/47	Not tested	6/47
10	6/47	Not tested	6/47
11	6/47	Not tested	6/47
12	6/47/81	Not tested	6/47/81
13	3A/3B/3C/71	3B/3C/71	3A/3B/3C/71
14	80/81	80/81	80/81
15	6/47/53/54/77/VA4	Not tested	6/47/53/54/77/VA4
16	52/52A/79/80	52/52A/79/80	52/52A/79/80
17	3A/3B/3C/71	3A/3B/3C/71	3A/3B/3C/71
18	79/53	79/53	79/53
19	3A/53	3A/53	3A/53
20	6/47/54/75/VA4	6/47/54/75/VA4	6/7/47/54/75/VA4
21	29/52/52A/80	29/52/52A/80	29/52/52A/80
22	52/80/81	52/80/81	52/80/81
23	42E/53/54	42E/53/54	42E/53/54
24	Untypable	Untypable	Untypable
25	42D/42E/52/77	Not tested	42D/42E/52/77

RESULTS

The phage patterns of 25 strains of coagulase-positive staphylococci isolated from the hospital environment and nasal cultures are presented in Table I. Table II shows the results obtained when 15 strains of staphylococci, previously typed by the conventional technique, were typed by the disk method. Results obtained by the microdroplet method and the reported patterns of the strains are presented in the tables for comparative purposes. The need for having an excess of phage particles incorporated into the disks is shown by the results obtained with disks containing approximately 10^3 particles.

Fig. 1 shows the test system as it is used in our laboratory and indicates the degree of contrast between positive and negative reactions.

TABLE II. PHAGE PATTERNS OF TYPED STAPHYLOCOCCAL STRAINS AS DETERMINED BY DISK TECHNIQUE

STRAIN	REPORTED PATTERN	PATTERN, DISK TECHNIQUE
31	6/7/47/53/54/75	6/7/47/53/54/75
32	6/47/54/75	6/47/54/75
33	6/47/53	6/47/53
34	6/7/47	6/7/47
35	52A/79	52A/79
36	29/52/52A/79/80	29/52/52A/79/80
37	3A/3B/3C/55	3A/3B/3C/55
38	80/81	80/81
39	29/52/80/81	29/52/80/81
40	29/52/52A/80/81	29/52/52A/80/81
41	3A/3B/3C/71	3A/3B/3C/71
42	53/54/77/VA4	53/54/77/VA4
43	80/81	80/81
44	47/53/54/75	47/53/54/75
45	29/52/52A/79	29/52/52A/79

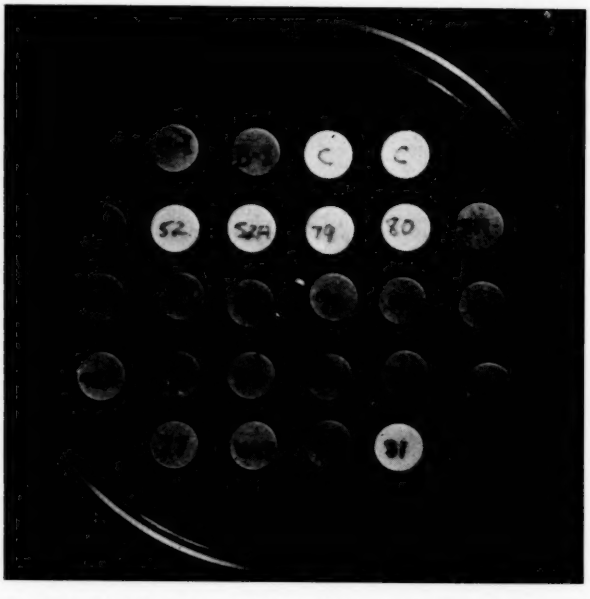

Fig. 1.—Photograph of the phage impregnated paper disk typing system showing the degree of contrast between positive and negative reactions 2 hours after inoculation. Top row contains control disks.

DISCUSSION

Conventional bacteriophage typing procedures are, in most instances, beyond the capability of the clinical laboratory. The propagation techniques for typing stocks are complicated and materials are expensive. For these and other reasons phage typing has been confined to a few large centers and research laboratories. The importance of phage typing as an epidemiologic tool, however,

demands that this procedure be used in the recognition and control of infections caused by drug-resistant staphylococci at the local level. Decisive corrective measures depend upon prompt recognition and identification of the causative organism before an outbreak reaches epidemic proportions. The development of phage impregnated disks with a color indicator system represents a simplification of the typing procedure and places this valuable epidemiologic tool within the capability of the clinical laboratory.

After the establishment of the suitability of redox indicators for the rapid visualization of the results of antibiotic and bacteriophage sensitivity tests, an attempt to design a test system utilizing paper disks appeared to be justified. Previous work has shown that impregnation of paper disks with bacteriophage was feasible.[14] The addition of a color indicator to the phage disks to produce a completely self-contained test system appeared to be desirable.

Phage disks containing tetrazolium chloride as a redox indicator system have been shown to be stable after storage for 60 days at room temperatures and for longer periods when stored under refrigeration. The preservation of the integrity of the phage disks in storage depends to a large extent upon maintaining a high degree of dehydration.

In an attempt to minimize the possibility of interaction between the redox indicator and the bacteriophages within the disks during preparation and storage, we are investigating the use of freeze-drying techniques.[21] Preliminary results indicate that disks prepared in this manner remain stable in storage for periods in excess of 90 days. We have also devised a phage disk utilizing a lamination technique in which each of the components is incorporated into separated disks. This test system is formed by placing the component containing phage on top of the component containing the redox indicator system. Mechanical separation of the two components is maintained during storage by a thin, porous tissue barrier. Upon inoculation of the laminated disk with broth the effectiveness of the barrier is lost and diffusion of the components occurs. The reaction between the bacterial cells and phages may then be observed by noting the color reaction of the system. Laminated phage disks have given excellent results in preliminary tests.

SUMMARY

Bacteriophage impregnated paper disks containing a redox indicator have been prepared and used in the typing of 40 strains of *Staphylococcus aureus*. The results obtained though the use of the phage disks were in excellent agreement with those obtained by conventional typing methods. The phage disk method represents an improvement in phage typing in that the disks can be prepared in advance of use, they may be stored, and the time required to obtain the sensitivity pattern is greatly reduced.

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A FLUOROMETRIC METHOD FOR THE DETERMINATION OF HIPPURIC ACID

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A method for determination of hippuric acid is described. It is based on the fluorescence of hippuric acid in 70 per cent sulfuric acid. Detailed procedure for urine analysis is presented. Evidence for the specificity of the reaction as applied to urine was obtained. Data on the accuracy and recoveries are also presented. The method is sensitive to final concentrations of 0.2 μg per milliliter.

THE classic method for determination of hippuric acid has been, since 1876, crystallization from urine, drying, and weighing.[1-3] Later modifications of this technique included tittrimetric procedures,[4-6] Kjeldahl analysis,[7] addition of salt for decrease of the solubility of the acid,[8] colorimetry,[9, 10] and the use of ether extraction curves.[11] The disadvantages include nonquantitative precipitation, impurity of the hippuric acid, and numerous correction factors which are involved in most of these procedures.

Recently, use has been made of ultraviolet spectrophotometry in the microdetermination of hippuric acid.[12, 13] Isolation by ion exchange chromatography[12] or differential ultraviolet spectrophotometry of benzoic and hippuric acids[13] has been proposed for quantitative analysis.

We have observed that hippuric acid in 70 per cent sulfuric acid fluoresces when irradiated with ultraviolet light, thus providing the basis for a sensitive, fluorometric determination. This method has several advantages over preceding ones: much smaller samples can be utilized, there is no appreciable loss of hippuric acid from the fluid being studied, greater sensitivity is afforded without the need for correction factors, and, for most urine determinations, no preliminary purification is necessary.

MATERIALS AND METHODS

Solutions.—

1. Seventy per cent sulfuric acid: Seven volumes of concentrated sulfuric acid* is added slowly to 3 volumes of deionized water, stirred, and cooled. When cool, keep in a Pyrex or polyethylene bottle which is tightly stoppered to prevent atmospheric water from entering.

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*Mallinckrodt Chemical Works, St. Louis, Mo. (A.R. grade) and General Chemical Division, Allied Chemical Company, New York, N. Y. (C.P.) sulfuric acids were found to be satisfactory.

2. Standard hippuric acid: 5 mg. per milliliter. Fifty milligrams of hippuric acid is weighed accurately. Approximately 30 mg. of sodium bicarbonate and exactly 10.0 ml. of deionized water are added. The hippuric acid will dissolve slowly. Keep refrigerated.

Instrument.—The fluorescence of the solutions was measured in an Aminco-Bowman spectrophotofluorometer; however, any fluorometer capable of exciting at 250 mμ and measuring at 375 mμ would be satisfactory.

Assays.—Prepare a series of test tubes by placing exactly 5.0 ml. of 70 per cent sulfuric acid into each. This is done easily with a 100 ml. burette equipped with a Teflon valve.

1. For the standard curve dilute the standard hippuric acid (Solution 2, above) 1:5. To the separate tubes, prepared as above, add 0, 2, 5, 10, 25, and 50 μl of this solution and mix well. These are then 0, 0.4, 1, 2, 5, and 10 μg per milliliter.

2. Duplicate samples of urine are usually run. A 2 μl aliquot is usually adequate for both pre- and postbenzoate injection samples.

Results are expressed as fluorescence intensity, which is the product of the reading of the transmittance scale and the meter multiplier setting.

Calculation.—From the standard curve determine the concentration of the hippuric acid in the urine dilution (c_1). Since the dilution was 2:5,000 = 2,500 fold, the concentration of hippuric acid in the urine is

$$c_0 \text{ (mg./ml.)} = 2{,}500\ c_1 \text{ (μg/ml.)} \times 10^{-3} \text{ mg./μg} = 2.5\ c_1$$

and the amount of hippuric acid excreted is

$$a \text{ (mg.)} = c_0 V \text{ (in milliliters)}.$$

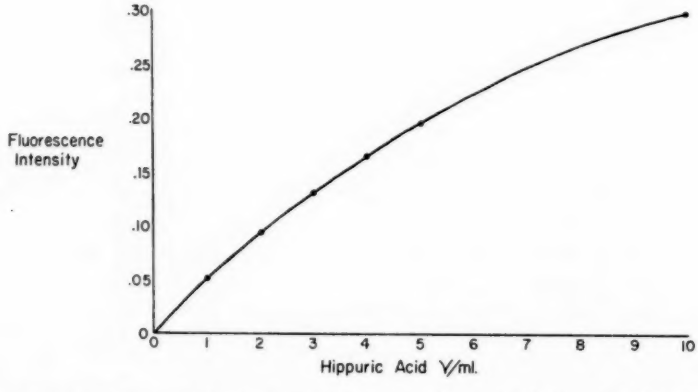

Fig. 1.—Standard curve: abscissa, final concentration of solution in micrograms per milliliter; ordinate, fluorescence intensity.

RESULTS

Standard Curve.—The result of several standard curve runs is shown in Fig. 1. The minimal significant concentration is approximately 0.2 μg per milliliter.

Fluorescent Spectra.—The excitation and fluorescent spectra of hippuric acid in 70 per cent sulfric acid are shown in Fig. 2. The fluorophor forms rapidly and is quite stable. We have been unable to follow the time course of its formation, since the fluorescence is formed as soon after mixing the reagents as we can get them in the fluorometer. We have noted no change in fluorescent readings of a solution over a 6-hour period.

Precision.—The reproducibility of the fluorescence method is shown in Table I. Six replicates of 3 different solutions were assayed for hippuric acid. The mean fluorescence intensity and standard deviation are given.

TABLE I. REPRODUCIBILITY OF THE FLUORESCENCE ASSAY METHOD

SOLUTION	μG PER MILLILITER HIPPURIC ACID	FLUORESCENCE INTENSITY ± STANDARD DEVIATION
Standard	2.0	0.125 ± 0.0033
Urine No. 1		0.131 ± 0.0050
Urine No. 2		0.221 ± 0.0084

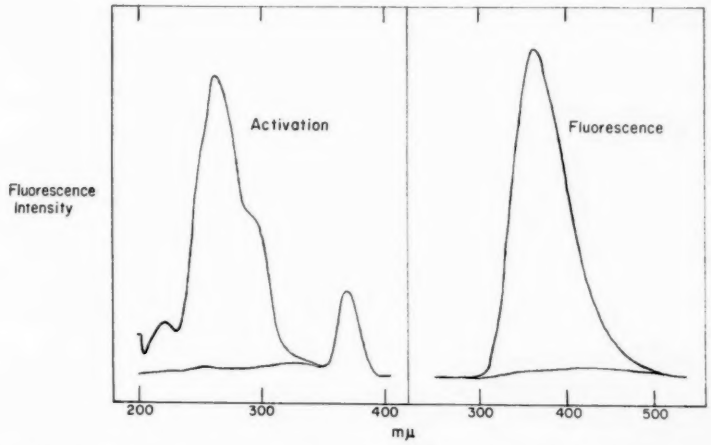

Fig. 2.—Activation and fluorescence spectra of hippuric acid (2 μg per milliliter) in 70 per cent sulfuric acid and corresponding spectra of 70 per cent sulfuric acid.

Specificity.—The specificity of this reaction was studied in two ways: (1) other compounds likely to interfere were dissolved in 70 per cent sulfuric acid and the solutions examined for fluorescence; (2) urines were chromatographed, the strips were cut into segments and eluted, and the eluates made up to 70 per cent sulfuric acid and examined for fluorescence.

In summary, we observed that all the benzoylated amino acids which were available to us (benzoyl-glycine, -glutamic acid, -alanine, and -tyrosine) fluoresced similarly and in approximately equivalent amounts. Also, benzoic acid and salicyluric acid fluoresced under these conditions. No amino acids fluoresced, nor did they interfere with the fluorescence of hippuric or benzoic acids. It would appear that the fluorophor is a product of the reaction of the aroyl group and sulfuric acid.

In order to establish that we were, indeed, measuring hippuric acid, urine samples were chromatographed in n-propanol-concentrated NH_4OH-H_2O (85:1:14). The chromatograms were scanned for ultraviolet absorbing materials at 250 mμ. These scans, as well as the 70 per cent sulfuric acid fluorophors, are shown in Fig. 3 as a function of R_f. It appears that most of the fluorescence of normal urine is due to hippuric acid, and, after an injection of sodium benzoate, hippuric acid accounts for over 95 per cent of

the fluorescence. It is possible for other benzoylated compounds to be detected by the fluorescence reaction, but no others have been observed on our chromatograms; the other available benzoylated compounds migrate to different areas of the chromatograms. Salicyluric acid migrates to the same general area as hippuric acid in these chromatograms; since it fluoresces without any treatment, its presence can be noted readily on chromatograms.

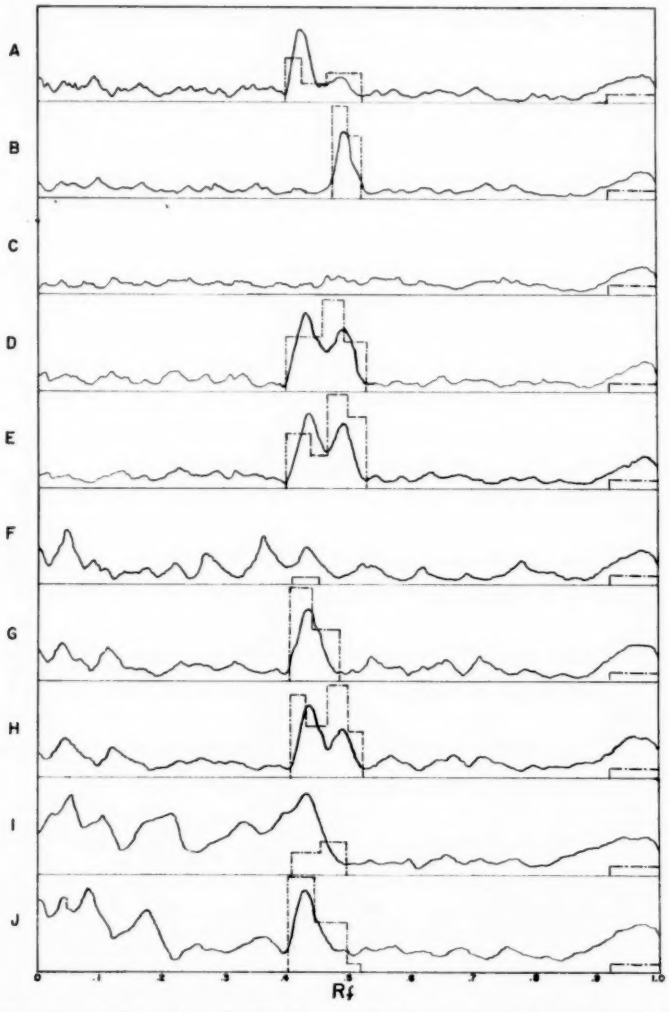

Fig. 3.—Ultraviolet absorption (solid line) and fluorescence (dashed lines) in 70 per cent sulfuric acid on chromatograms as a function of Rf: *A*, hippuric acid; *B*, benzoic acid; *C*, glycine (located by treatment with Ninhydrin at Rf 0.12); *D*, hippuric and benzoic acids; *E*, glycine, hippuric, and benzoic acids; *F*, urine, Subject A, before benzoate injection; *G*, urine, Subject A, after benzoate injection; *H*, urine, Subject A, after benzoate injection, with benzoic acid added to the chromatogram; *I*, urine, Subject B, before benzoate injection; *J*, urine, Subject B, after benzoate injection.

Urine Analysis.—A few samples of urine were analyzed by both fluorometry and the precipitation methods. The comparison is shown in Table II. Note that small amounts of hippuric acid tend to coprecipitate with other

materials, giving high values, whereas larger amounts give low values, probably because of solubility in urine. The values obtained from normal urine samples range from 0.3 to 3.2 mg. per milliliter. This can be extrapolated to 0.3 to 3.2 Gm. per day for a 1,000 ml. daily excretion. The daily normal excretion values are given as 0.1 to 1.0 (usually 0.5 to 0.7 Gm.) daily.[14] Since much of the normal excretion may come from quinic acid metabolism,[15] the urinary excretion is probably dependent on the diet of the individual.

TABLE II. URINARY HIPPURIC ACID CONTENT: COMPARISON OF TWO METHODS

SOLUTION	FLUORESCENCE (GM.)	PRECIPITATION (GM.)
Urine A.—		
Prebenzoate	0.071	0.139
Postbenzoate	1.423	1.172
Urine B.—		
Prebenzoate	0.082	0.183
Postbenzoate	0.686	0.333

TABLE III. RECOVERY EXPERIMENTS

SOLUTION	HIPPURIC ACID (μG/PER MILLILITER)			RECOVERY (PER CENT)
	ORIGINAL	ADDED	RECOVERED	
Urine No. 1	1.20	0.40	1.55	98
Urine No. 2	4.55	0.40	5.00	101

Recovery.—Data from recovery experiments are shown in Table III. Clearly, recovery is quantitative within the limits of the other experimental variables (pipetting, fluorometry, etc.).

DISCUSSION

No attempts have been made to apply the method to serum. We have run a few dozen urine samples without observing any complications. However, dietary constituents, drugs, etc., may interfere. We feel that chromatography, as we have used it, can be helpful in eliminating any suspected complications. In addition, it has been most useful to determine prebenzoate excretion values of hippuric acid; some individuals appear to excrete appreciable quantities.

The correlation of anxiety and hippuric acid excretion in humans which was described by Persky and associates[16] was studied in our laboratory.[17] Hippuric acid was measured by the fluorometric method, and the values obtained were in essential agreement with those presented by Persky, who used a precipitation method.

SUMMARY

The fluorescence of hippuric acid in 70 per cent sulfuric acid has been used as the basis of a quantitative assay. Small samples of urine (2 μl) are required. For determinations after sodium benzoate injection, no purification of the urine is required. Specificity studies have indicated that the fluorescence is due to the aroyl component of the hippuric acid; thus other benzoyl derivatives would interfere if present. However, chromatographic studies of urine have revealed no appreciable amounts of interfering materials.

Recovery experiments and comparison with the precipitation method indicate that the fluorometric method is quantitative, shows less variability, and is technically simpler than the precipitation technique.

We wish to thank Dr. A. N. Booth of the laboratory of the Western Regional Department of Agriculture for the supply of salicyluric acid.

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Correction

Confusion has arisen concerning the reagent employed for total lipid analysis (A Simple Method for Determination of Total Lipids in Serum, by M. Brandstein and A. Castellano, J. Lab. & Clin. Med., vol. 57, p. 300, February, 1961).

The correct reagent has been given the name Lipitest reagent (Merck & Co., Inc.), and differs in composition from the Schain test reagent used in butterfat determinations.

A DIRECT METHOD FOR THE QUANTITATIVE MEASUREMENT OF RED CELL DIMENSIONS

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Quantification of erythrocyte constituents has limited usefulness if the constituents are regarded as a function of the unit of weight or volume of cells. This becomes a consideration of particular importance if factors such as the variation in size and shape of normal and especially of abnormal cells, the uneven distribution of cell constituents, and the surface localization of certain components are recognized. Thus, a simple, accurate technique for measurement of cell dimensions would be helpful.

A microprojection technique in which direct measurements of cell dimensions are made is described. Measurements of normal cells in wet preparations, both singly and in rouleau formation, and of normal cells in dry stained smears were made in 10 normal individuals. The results were tested for reproducibility and compared to measurements of other investigators in which photomicrographic or indirect techniques were used.

The mean diameter and thickness of cells in rouleaux in wet preparations were 8.70 $\mu \pm 0.29$ and 1.64 $\mu \pm 0.07$, respectively. The diameter of single flat cells in wet preparations was 8.56 $\mu \pm 0.21$, and the mean diameter of single cells in dry stained smears was 7.11 $\mu \pm 0.40$. Mean cell volume was 86.1 $\mu^3 \pm 6.3$ and mean cell surface area 145.0 $\mu^2 \pm 8.3$. A repeat series of measurements revealed the mean diameter and thickness to be 8.61 $\mu \pm 0.20$ and 1.58 $\mu \pm 0.09$, respectively, in comparison to earlier measurements for diameter and thickness of 8.69 $\mu \pm 0.20$ and 1.64 $\mu \pm 0.07$, respectively. Comparison between techniques demonstrated that the results of the present technique were most similar to those obtained by Ponder with a photomicrographic method.

VARIATIONS in the size and shape of normal erythrocytes may impose serious limitations upon the interpretation of the quantification of certain red cell components. This is particularly true if abnormal forms are being measured or if concentrations of materials are considered as a function of the individual cell rather than as a function of the unit of weight or volume of cells. Difficulties may become even more complex if the components are unevenly distributed throughout the cell or have primary associations with subcellular entities, such as the stroma, or with cell parameters, such as surface area.

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Earlier approaches to this problem have been to obtain the cell measurements by photomicrography or other means, and from these dimensions to calculate the volume and surface area of the cell. The many inherent problems, as well as a critical evaluation of attempts to obviate the difficulties and simplify the techniques, have been described by Ponder.[1] However, even with the availability of very fine and precise optical systems, questions concerning the desirability of photomicrographic rather than direct measurement, and uncertainties of single cell measurement rather than that of cells in rouleaux are in many instances not completely defined.

Evaluation of these measurement techniques requires comparison between the results of the unproved technique and those of an absolute standard in which there are no defects. This would be particularly true in the measurement of the erythrocyte, since it can be measured in several forms, such as the single cell, the cell in rouleaux, or the cell in the horizontal or vertical positions. Dependent upon form, dimensions may be subject to variable alterations and distortions. For the most suitable morphologic cell standard, the measured cells should be freely suspended in physiologic media, preferably native plasma, and nonphysiologic external forces or stimuli should be absent. These abnormal forces would include not only the possible distorting effect which might occur with the use of nonphysiologic suspending media or by the effects of rouleau formation on cell size, but also the possible distorting collapsing effect which might occur if single cells standing on edge were used as the form for cell measurement. In addition, preparations should not contain properties which might alter or variably distort the cell, such as the shrinkage which has been noted to occur in stained dried preparations. The above factors represent a few of the several conditions necessary for adequate determination of cell size and some of the major difficulties in the formulation of an ideal standard cell form for measurement. Consequently, the necessarily acceptable standard for comparison will not be identical with the ideal standard. The evaluation of a technique for measurement of red cell size, therefore, may contain two separate and distinct variables, i.e., the optical portion of the system, and the form of the cell used for measurement. This, as can be readily realized, may cause numerous interpretative difficulties.

Further definition of these problems, as well as a simple, yet precise, practical, reproducible technique for cell measurement is highly desirable. We have utilized a nonphotographic microprojection technique in which direct measurements are made of cells in rouleaux in wet preparations. To evaluate this technique, and to clarify some of the problems cited above, attempts have been made to define reproducibility and accuracy as these relate to data obtained by other methods of measurement. To diminish the problem of two variables, measurements of the flat single cell in native plasma were made and the results compared to those obtained by the indirect methods of other investigators. The determination of this quite simple cell dimension minimizes one variable and thus provides a suitable basis for comparison of techniques. For adequate comparison of cell dimensions as determined in various types of cell preparations

we have used measurement forms which were similar to those used by the various authors in their techniques, e.g., rouleau cells were compared to rouleau cells, and flat cells to flat cells.

METHODS

Wet preparations of erythrocytes were made after collection of blood by venipuncture with the use of the disodium salt of ethylenediaminetetraacetic acid (1 mg. per 10 ml. of blood) as the anticoagulant. The specimens of blood were then centrifuged (RCF = 1,000) for 10 minutes, the plasma removed, and a suspension of red cells in native plasma prepared. The usual concentration used was approximately 1 per cent. However, the adequacy of any preparation was readily and finally determined by microscopic examination of a diluted wet preparation of the erythrocytes. A cover slip preparation, in which the cells appeared well distributed and in which there were no crenation, motion, or artifactually altered cell surfaces, was made, sealed with a suitable material, such as petroleum jelly, and projected onto a calibrated screen for cell measurements.

The microprojection apparatus consisted of the microscope (Leitz Ortholux) and projection prism, mounted on a rigid metal frame. A xenon light source was used. The objective lens was an Apochromat with oil immersion 90:1, 170/0.17, and numeric aperture 132. A 10× ocular lens was used. The magnification provided by this system was 2,600. The cell dimensions were measured by the use of calipers and a calibrated scale. The scale was prepared from the projection of a 0.2 mm. ruled stage micrometer scale (Leitz) onto a screen on which a careful, precise tracing was made. The accuracy and reproducibility of the system were determined for each series of measurements by projection of the micrometer scale onto the previously prepared calibrated scale. In practice, however, there was little or no variability when stable and fixed equipment was used. After location of desirable cells for measurement, more precise adjustments for clarification of cell edges, contrast, and for diminishment of glare were then made.

Measurements were made on at least 100 cells in each preparation. In general, the basis for selection of cells for measurement was relative freedom from external pressures. In the measurement of cells in rouleaux, columns of 7 to 15 cells were used and the two end cells not measured. Three different observers performed the measurements.

Cell volume and cell surface area were calculated by relating the cell to a spheroid and utilizing the formulas:

$$V\ (\mu^3) = 0.0712 \times d^2 \times t$$

where d = diameter and t = thickness and

$$SA\ (\mu^2) = 2\Pi a^2 + 2\Pi ab \cdot \sinh^{-1} e/e$$

in which a = radius, b = 0.67 × thickness, and $e = \sqrt{a^2 - b^2/a}$

Measurements were made in 30 separate specimens of red cells obtained from ten healthy individuals, five men and five women who were in a postabsorptive state and between the ages of 20 to 40. Cells in rouleaux and single cells, usually from the same wet preparation, were measured. Individual cell diameters were also measured in dry smears stained with Wright's stain. Of the 30 measurements made, 15 were repeated approximately one month after the initial study, under circumstances identical to those of the original measurement.

RESULTS

The mean diameter of red cells in rouleaux in wet preparations as measured by this technique was 8.70 μ ± S.D. 0.29. Mean cell thickness was 1.64 μ ± 0.07. Cell volume and cell surface area were 86.1 μ^3 ± 6.3 and 145.0 μ^2 ± 8.3, respectively. These results are presented in Table I.

The mean diameter of single flat cells, measured in the same preparations as described above, was 8.56 $\mu \pm 0.21$. The mean diameter of single flat cells in stained dried smears was 7.11 $\mu \pm 0.40$. These are shown in Table II.

TABLE I. RED CELL DIMENSIONS AS MEASURED IN TEN NORMAL INDIVIDUALS*

SUBJECT	DIAMETER (μ)	THICKNESS (μ)	SURFACE AREA (μ^2)	VOLUME (μ^3)
E. S.	8.37	1.73	136.9	84.9
H. G.	8.25	1.62	131.6	77.3
M. W.	8.61	1.71	143.7	88.7
S. M.	8.58	1.50	140.0	77.4
L. P.	8.83	1.64	149.7	88.9
R. A.	8.80	1.63	149.0	88.4
N. L.	8.77	1.57	147.1	84.6
A. B.	8.60	1.59	141.6	82.4
B. S.	9.03	1.58	154.4	89.7
J. M.	9.14	1.68	159.7	98.4
Mean	8.70 ± 0.29	1.64 ± 0.07	145.0 ± 8.3	86.1 ± 6.3
Range	8.25-9.14	1.50-1.73	131.6-159.7	77.3-98.4
S.E.	0.09	0.02	2.68	1.98

± Refers to standard deviation.
*Measurement of cells in rouleaux.

TABLE II. MEAN RED CELL DIMENSIONS REPORTED BY VARIOUS AUTHORS

SOURCE OF INFORMATION	DIAMETER (μ)				THICKNESS (μ)		SURFACE AREA* μ^2	VOLUME* μ^3
	SINGLE CELL							
	FLAT							
	WET	DRY	ON EDGE	ROULEAUX	SINGLE CELL ON EDGE	ROULEAUX		
Ponder and associates[5]	8.4	6.9-7.8						
Ponder[1, 2]	8.65		8.55	8.83	1.71	1.71	155†	87
Houchin and associates[3]				8.28		1.71	134†	82
Bingham[4]		7.14						
Present	8.56	7.11		8.70		1.64	145†	86

*Values calculated by individual authors.
†Derived considering the cell as a spheroid.

TABLE III. INITIAL AND REPEAT ERYTHROCYTE MEASUREMENTS* ON FIFTEEN CELL PREPARATIONS

	DIAMETER (μ)	STANDARD ERROR	THICKNESS (μ)	STANDARD ERROR
Initial	8.69 ± 0.20	0.052	1.64 ± 0.07	0.018
Repeat	8.61 ± 0.20	0.051	1.58 ± 0.09	0.024
P value	0.3		0.08	

± Refers to standard deviation.
*Rouleau formation in native plasma.

The results of repeat measurements on 15 of the original 30 examinations can be seen in Table III. The repeat mean cell diameter and thickness were 8.61 $\mu \pm 0.20$ and 1.58 $\mu \pm 0.09$, respectively, in comparison to initial measurements for this group of 8.69 $\mu \pm 0.20$ and 1.64 $\mu \pm 0.07$.

DISCUSSION

The diameter of the single flat cell suspended in plasma, which is theoretically the closest to the ideal form of cell preparation, was measured and

compared to the results obtained by Ponder,[2] in whose experiment a photomicrographic technique of similarly suspended cells was used. Results show the mean diameter to be 8.65 μ by Ponder's technique and 8.56 $\mu \pm 0.21$ by the present direct method. Since this measurement provides comparable cell forms in suitable cell media, any difference in size essentially represents optical differences and thus gives a better basis for comparisons of techniques. It would appear that the differences between the two systems is quite small. This may be seen in Table II.

Further comparison with Ponder's study[2] reveals the mean diameter and thickness of cells in rouleaux to be 8.83 μ and 1.71 μ, respectively, as measured in 5 specimens by his methods. Measurements with similar rouleaux suspensions, as determined by the present technique, reveal a diameter of 8.70 $\mu \pm 0.29$ and thickness of 1.64 $\mu \pm 0.07$. Thus, as shown in Table II, both studies reveal that cells in rouleaux exhibit greater diameters than do flat cells. At the same time, the studies demonstrate a small difference between measurements obtained by the two methods. Explanation of this difference is not completely satisfactory, although statistical examination of comparable values reveals definite overlap.

A recent study in red cell measurement has been that of Houchin and coworkers,[3] in which cells in rouleaux suspended in plasma were measured by a photomicrographic technique. Since comparisons between results of the 2 techniques must be made on measurements of cells in rouleaux, there are added variables which do not yield as close primary comparisons of optical methods as does the comparison with Ponder's measurements. They reveal, however, measurements of 8.28 μ and 1.71 μ for diameter and thickness, respectively. Thus, the diameter is smaller than in the present study (8.70 μ) while the thickness determination is larger than that observed in the present study (1.64 μ). These are demonstrated in Table II. One implication from these results would be that variable forces which are altering cell dimensions are being exerted upon the cells. A factor in measurements such as this may be rouleau length or the type of cell column measured.

Bingham[4] has measured the diameter of dry stained erythrocytes with the use of a direct microprojection technique. The cells were then outlined with pencil and measured. The results (7.14 μ) were very similar to those seen in the present analysis (7.11 μ) and to those made by Ponder[5] in an earlier study. In all studies there was a distinct decrease in cellular size in the dry stained preparations as compared to that of the wet preparation.

Reproducibility of the results obtained with this method requires analysis. To evaluate this, 15 of the measurements of rouleau wet cells were repeated at a later date on fresh samples and the measurements for diameter and thickness found to be 8.61 $\mu \pm 0.20$ and 1.58 $\mu \pm 0.09$, respectively, against earlier measurements of 8.69 $\mu \pm 0.20$ and 1.64 $\mu \pm 0.07$, respectively. P values were 0.3 and 0.08 for the corresponding diameter and thickness determinations. These results may be seen in Table III. In consideration of technical and biologic variation in the same individual, the results seem quite similar and the technique appears reproducible.

Certain aspects of the data require further consideration. Comparison between the variation inherent in the type of optical system or cell preparation used and the biologic variability of erythrocyte dimensions would be helpful for evaluation of the significance of the differences observed in various methods. The similarity of results obtained by different methods is evident in the diameter measurements, as recorded by Ponder,[2] of the flat single cell (8.65 μ), of the cell on edge (8.55 μ), or of the cell in rouleaux (8.83 μ), and may be observed in the diameter measurements as determined by the present technique of the flat single cell (8.56 μ) or of cells in rouleaux (8.70 μ). The biologic variation of the diameter of the normal cell is demonstrated by examination of the range of measurements of the normal cell and by the magnitude of the standard deviation of the measurements. The latter factor, which is a measure of the spread about the mean, would represent normal variation. The range of dimensions and the standard deviation as shown by Ponder[2] for "on edge" cell measurements were 8.40 to 8.67 μ, S.D. $\pm$ 0.41 μ, for dry stained cells shown by Bingham[4] they were 6.80 to 7.49 μ, S.D. $\pm$ 0.34 to 0.56 μ, and for cells in rouleaux as observed by the present technique they were 8.25 μ to 9.14 μ, S.D. $\pm$ 0.29 μ. Thus, it is evident that variation between techniques is less than the normal biologic variation of measurements. This would minimize the apparent differences in results observed between methods.

Finally, there are several features of this system which would indicate that the described technique is practicable and inherently adequate for the determination of erythrocyte dimensions. Considerations, such as the omission of photography, the ability to focus individually each cell, the ability to locate in the center of each field the cell or group of cells being measured, the relative speed of the procedure, with subsequent decreased propensity for in vitro cellular alteration, and the relatively lower cost of the system are all advantages in this measurement technique.

SUMMARY

A technique of direct microprojection of cells is described for use in the determination of red cell dimensions. The method appears to be accurate and reproducible and at the same time simpler, more practicable, and less costly than other available techniques. Cells in rouleau formation have altered dimensions as compared to cells measured in a more physiologic state. Cells in wet preparations are larger than those in dry preparations.

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